



520

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁶ : A61K 38/00, C07K 2/00, 4/00, 5/00, 7/00, 14/00, 16/00, 17/00, C12Q 1/00</p>	<p>A1</p>	<p>(11) International Publication Number: WO 98/22120 (43) International Publication Date: 28 May 1998 (28.05.98)</p>
<p>(21) International Application Number: PCT/US97/21116 (22) International Filing Date: 19 November 1997 (19.11.97) (30) Priority Data: 60/031,169 19 November 1996 (19.11.96) US (71) Applicants (for all designated States except US): THE WISTAR INSTITUTE OF ANATOMY & BIOLOGY [US/US]; 3601 Spruce Street, Philadelphia, PA 19104 (US). TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA [US/US]; Suite 300, 3700 Market Street, Philadelphia, PA 19104 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): OTVOS, Laszlo [US/US]; 801 Mockingbird Lane, Audubon, PA 19403 (US). HOFF- MANN, Ralf [DE/US]; 6812 Drexelbrook Drive, Drexel Hill, PA 19026 (US). LEE, Virginia, M-Y. [US/US]; 2005 Pine Street, Philadelphia, PA 19103 (US). (74) Agents: KODROFF, Cathy, A. et al.; Howson and Howson, Spring House Corporate Center, P.O. Box 457, Spring House, PA 19477 (US).</p>		<p>(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: DIAGNOSTIC AND THERAPEUTIC REAGENTS FOR ALZHEIMER'S DISEASE (57) Abstract A synthetic multiphosphorylated peptide derived from the τ protein of the paired helical filaments is described. The Alzheimer disease specificity is provided by the presence of phosphate moieties on nearby serine and threonine residues, and the reverse-turn structure of the multiphosphorylated protein fragment. The multiphosphorylated peptide is useful as an antigen and a binding partner for identifying inhibitor compounds which interact with the peptide and the hyperphosphorylated tau protein, including Alzheimer's specific antibodies. The resulting antibodies are useful diagnostically and therapeutically. The inhibitors that specifically bind to multiphosphorylated tau peptides and proteins are useful for eliminating abnormally hyperphosphorylated tau.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

DIAGNOSTIC AND THERAPEUTIC REAGENTS FOR ALZHEIMER'S DISEASE

This invention was supported by the National Institutes of Health grants AG10670 and AG10210. The US government has certain rights in this invention.

Field of the Invention

The present invention relates generally to compositions useful in diagnosis of Alzheimer's disease, methods of producing same, and structural requirements for inhibitor design.

10 Background of the Invention

The major histopathological abnormalities that characterize the brains of patients with Alzheimer's disease (AD) as well as those of older individuals with Down syndrome include excesses of neurofibrillary tangles (NFT), and senile plaques (SP) (K. S. Kosik, *Science* 256:780-783 (1992); V.M.-Y. Lee and J. Q. Trojanowski, 15 *Curr. Opin. Neurobiol.*, 2:653-656 (1992); D. J. Selkoe, *Trends Neurosci.*, 16:403-409 (1993)]. The NFT are composed of paired helical filaments (PHF) that are biochemically and structurally distinct from the amyloid fibrils in SP. PHF and the straight filaments that dominate ultrastructural images of neurofibrillary lesions are likely formed from hyperphosphorylated forms of the low molecular weight 20 microtubule-associated τ protein, known as PHF- τ [M. Goedert, *Trends Neurosci.*, 16:460-465 (1993); J. Q. Trojanowski et al, *Clin. Neurosci.*, 1:184-191 (1994)]. In contrast, the major subunit proteins of amyloid fibrils are β -amyloid peptides (A β). They are about 42 amino acids long, and are derived from one or more larger, alternatively spliced amyloid precursor proteins (APP), encoded by a gene on 25 chromosome 21 [J. Hardy, *J. NIH Res.*, 5:46-49 (1993); M. Mullan and F. Crawford, *Trends Neurosci.*, 16:409-414 (1993)]. SP are sites at which abundant accumulations of both PHF and amyloid fibrils converge (Trojanowski et al., 1994, cited above). Despite the presence of APP mutations in rare cases of familial AD, the abundance of

NFT in the brain correlates better with dementia severity in AD than the density of amyloid plaques [D. W. Dickson et al, Neurobiol. Aging, 13:179-189 (1991); P. A. Arriagada et al, Neurology, 42:631-639 (1992)].

5 A major prerequisite for evaluating therapeutic and/or prevention strategies of AD, the availability of valid biological markers of AD is still lacking. In this regard, it is significant that τ levels are increased in the cerebrospinal fluid (CSF) of AD patients and that the CSF τ levels correlate with clinical measures of dementia severity [C. Mock et al, Ann. Neurol., 37:414-415 (1995)]. The assay used the very sensitive monoclonal antibody (mAb) AT120 for detection of τ . Unfortunately, mAb
10 AT120 detects normal τ and PHF- τ just as well. Most recently, tau was detected in humans. The development and use of more specific antibodies that are both highly sensitive, and can distinguish between normal and deranged τ , may further enhance the discriminative properties of such assays and seems likely to be the focus of research in the forthcoming years.

15 The sites of abnormal phosphorylation of PHF- τ and the structural changes abnormal phosphorylation bring, are two of the most studied and debated areas of the current AD research. Functionally, τ binds to tubulin and PHF- τ does not [C. W. Scott et al, J. Neurosci. Res., 33:19-29 (1992); Biernat et al., Neuron, 11:153-163 (1993)]. The lack of the microtubule binding of PHF- τ is usually attributed to
20 hyperphosphorylation as tubulin binding can be restored by dephosphorylating PHF- τ [P. Seubert et al., J. Biol. Chem., 270:18917-18922 (1995)]. There is some concern that, after several years of intense analysis of the phosphorylation status of τ , there is still no compelling evidence that phosphorylation of τ has any causal relation to the formation of PHFs. The views opposing hyperphosphorylated τ as a primary reason
25 for PHF production come from the observation that constructs corresponding roughly to the microtubule-binding repeat region of τ can form synthetic PHFs. A similar self-assembly occurs with chemical cross-linking of dimers. In both cases, there is no need for phosphorylation of the protein. The absence of abnormal hyperphosphorylation of τ in intracellular tangles of AD was also noted by studying the staining of NFT with
30 supposedly phosphate-specific mAbs, such as AT8, AT180, AT270, SMI31, Alz50

and BR133 [W. Bondareff et al., J. Neuropathol. Exp. Neurol., 54:657-663 (1995)]. In reality, however, these antibodies may not be ultimately specific for PHF (Goedert et al., 1994, cited above). The conclusive test whether an antibody is truly specific for PHF is the antibody's ability to label fetal τ and biopsy-derived τ . Fetal τ and biopsy-derived τ were shown to contain more phosphate groups than normal τ [T. J. Singh et al., Arch. Biochem. Biophys., 328:43-50 (1996); Garver et al., J. Neurosci. Res., 44:12-20 (1996)]. Dephosphorylation of PHF- τ can be very fast at some sites (Seubert et al., 1995, cited above), giving rise to the possibility of unrealistic immunoreactions with antibodies directed against these sites. Biopsy-derived τ refers to τ preparations from healthy individuals when the protein is isolated from the brain tissue seconds after acquisition, and τ is not exposed to the extremely fast phosphatase actions that occur in the tissues even post-mortem.

An increasing amount of evidence suggests that mAbs obtained after immunization with PHF- τ recognize multiphosphorylated protein fragments. The major recognition site of mAb PHF-1 is phosphorylated Ser396, but the recognition is increased when Ser404 is also phosphorylated [Otvos et al., J. Neurosci. Res., 39:669-673 (1994)]. Another often used antibody to PHF- τ is AT8 (Biernat et al., cited above). This mAb binds specifically to phosphorylated Ser202 and Thr205. As the previous investigations used singly phosphorylated synthetic peptides or mutated protein constructs to identify the binding sites of these mAbs, the close-to-minimal or minimal epitopes and exact phosphate requirements of the antibodies have not been characterized.

What is needed in the art are diagnostic antibodies that distinguish PHF- τ from other τ forms such as biopsy-derived τ , fetal τ and normal τ . Also needed is an understanding of how phosphorylation changes the conformation of tau and its fragments in order to design compounds that are able to binding hyperphosphorylated tau and remove it once it is formed.

Summary of the Invention

In one aspect, the invention provides a synthetic multiphosphorylated peptide derived from the tau protein of the paired helical filaments (PHF) associated with Alzheimer's disease. The multiphosphorylated peptide is useful as an immunogen
5 for preparing an antibody specific for Alzheimer's disease.

In another aspect, the present invention provides a method for generating one or more antibodies useful in the specific diagnosis of Alzheimer's disease. The method involves at least the step of administering to a mammal a synthetic multiphosphorylated peptide of the invention.

10 In yet another aspect, the present invention provides one or more antibodies generated according to the method of the invention. The resulting antibodies are characterized by specificity for the tau protein of the paired helical filaments associated with Alzheimer's disease.

In a further aspect, the present invention provides a method of treating
15 a patient with Alzheimer's disease comprising the step of administering to the patient a reagent of the invention. Preferably, this reagent is an antibody.

In still a further aspect, the present invention provides a method of treating a patient with Alzheimer's disease comprising the step of administering to the patient a multiphosphorylated peptide of the invention. Suitably, this generates anti-
20 peptide antibodies that are able to eliminate abnormally hyperphosphorylated tau peptide.

In yet a further aspect, the present invention provides for the use of a multiphosphorylated peptide of the invention in designing and identifying compounds useful in the diagnosis and treatment of Alzheimer's disease.

25 Other aspects and advantages of the present invention are described further in the following detailed description of the preferred embodiments thereof.

Brief Description of the Figures

Fig. 1 illustrates the selection of PHF-specific antibodies by ELISA. The diagonally hatched bars indicate the recognition of the newly developed mAbs against normal τ . The cross-hatched bars indicate the recognition of the mAbs against A68, putative AD-specific proteins, and purified forms of PHF (Lee et al., 1991). The single asterisks indicate the mAbs selected for further studies. PHF11 was selected because high background was obtained and (based on the first screening) its PHF-specificity could not be established (double asterisk).

Fig. 2A illustrates the binding of mAb PHF-1 to different tau preparations on Western blot. Lanes 1 and 2 are normal adult autopsy-derived τ , lanes 3 and 4 are fetal τ , lanes 5 and 6 are PHF- τ .

Fig. 2B illustrates the binding of mAb PHF-6 to different tau preparations on Western blot. Lanes 1 and 2 are normal adult autopsy-derived τ , lanes 3 and 4 are fetal τ , lanes 5 and 6 are PHF- τ .

Fig. 2C illustrates the binding of mAb PHF-13 to different tau preparations on Western blot. Lanes 1 and 2 are normal adult autopsy-derived τ , lanes 3 and 4 are fetal τ , lanes 5 and 6 are PHF- τ .

Fig. 2D illustrates the binding of mAb PHF-27 to different tau preparations on Western blot. Lanes 1 and 2 are normal adult autopsy-derived τ , lanes 3 and 4 are fetal τ , lanes 5 and 6 are PHF- τ .

Fig. 3 illustrates peptide recognition of mAb PHF-6. The ELISA plate was coated with 8 ng - 1 μ g antigens dissolved in water. Solid line: unphosphorylated peptide τ 224-240 [SEQ ID NO: 8]; dashes: peptide phosphorylated on Thr231 [SEQ ID NO: 9]; dots: peptide phosphorylated on Ser235 [SEQ ID NO: 10]; dots and dashes: peptide phosphorylated on both residues [SEQ ID NO: 11]. Undiluted supernatant of cells producing the antibody were used.

Fig. 4 illustrates peptide recognition of mAb PHF-27. The ELISA plate was coated with 4 ng- 2 μ g antigens dissolved in water. Solid line: unphosphorylated peptide τ 224-240 [SEQ ID NO: 8]; dashes: peptide phosphorylated on Thr231 [SEQ ID NO: 9]; dots: peptide phosphorylated on Ser235 [SEQ ID NO: 10]; dots and

dashes: peptide phosphorylated on both residues [SEQ ID NO: 11]. Undiluted supernatant was used.

Fig. 5 provides the identification of the epitope for mAb AT10. The ELISA plate was coated with 70 ng - 5 μ g monophosphorylated or diphosphorylated 208-222 peptides dissolved in water. The mAb was diluted to 1:300. Only the Thr 212, Ser214 diphosphorylated peptide was recognized by mAb.

Fig. 6A illustrates the dependence of antibody binding upon the conformation of the antigens, where the primary antibody is PHF-6. The peptides were applied to the ELISA plate in water or TFE, and dried to the plastic. Solid line: Thr231 monophosphorylated peptide applied from water [SEQ ID NO: 9]; dots: the same peptide applied from TFE. Dashes: Thr231, Ser235 diphosphorylated peptide applied from water [SEQ ID NO: 11]; dots and dashes: the same peptide applied from TFE. Many of the curves show the characteristic "pro-zone" antibody binding at high antigen loads, illustrating the close-to-optimal antigen-antibody interactions.

Fig. 6B illustrates the dependence of antibody binding upon the conformation of the antigens, where the primary antibody is PHF-27. The ELISA was performed as described in the legend to Fig. 6A.

Fig. 7 illustrates the dependence of antibody binding upon the conformation of the antigens where the primary antibodies are TG3 and AT180. The curves are as follows: Solid lines represent the Thr231, Ser235 double phosphorylated peptide [SEQ ID NO: 11] applied from TFE; dots and dashes represent the same peptide applied from water. Dashes represent the Thr231 phosphorylated peptide [SEQ ID NO: 9] applied from TFE; dots represent the same peptide applied from water. The ELISA was performed as described in the legend to Fig. 6A.

Fig. 8A provides the CD spectra of τ 224-240 peptides in water. Solid line: unphosphorylated peptide [SEQ ID NO: 8]; dots: Thr231 phosphorylated peptide [SEQ ID NO: 9]; dashes: Ser235 phosphorylated peptide [SEQ ID NO: 10]; dots and dashes: diphosphorylated peptide [SEQ ID NO: 11].

Fig. 8B provides the CD spectra of τ 224-240 peptides in TFE. Solid line: unphosphorylated peptide [SEQ ID NO: 8]; dots: Thr231 phosphorylated

peptide [SEQ ID NO: 9]; dashes: Ser235 phosphorylated peptide [SEQ ID NO: 10]; dots and dashes: diphosphorylated peptide [SEQ ID NO: 11].

Fig. 9A illustrates low energy conformers of the peptide backbone of unphosphorylated peptide τ 224-240 [SEQ ID NO: 8]. High dielectric constant was used to mimic solvent effects (78 for water for TFE). Unconstrained simulated annealing calculations were performed as described herein. The structures were overlaid automatically based on the similarity of all backbone atoms.

Fig. 9B illustrates low energy conformers of the peptide backbone of unphosphorylated peptide τ 224-240 [SEQ ID NO: 8]. Low dielectric constant was used to mimic solvent effects (4.5 for TFE). Unconstrained simulated annealing calculations were performed as described herein. The structures were overlaid automatically based on the similarity of all backbone atoms.

Fig. 9C illustrates low energy conformers of the peptide backbone of monophosphorylated peptide Thr231 [SEQ ID NO: 9]. High dielectric constant was used to mimic solvent effects (78 for water). Unconstrained simulated annealing calculations were performed as described herein. The structures were overlaid automatically based on the similarity of all backbone atoms.

Fig. 9D illustrates low energy conformers of the peptide backbone of monophosphorylated peptide Thr231 [SEQ ID NO: 9]. Low dielectric constant was used to mimic solvent effects (4.5 for TFE). Unconstrained simulated annealing calculations were performed as described herein. The structures were overlaid automatically based on the similarity of all backbone atoms.

Fig. 9E illustrates low energy conformers of the peptide backbone of diphosphorylated analogue of peptide τ 224-240 [SEQ ID NO: 11]. High dielectric constant was used to mimic solvent effects (78 for water for TFE). Unconstrained simulated annealing calculations were performed as described herein. The structures were overlaid automatically based on the similarity of all backbone atoms.

Fig. 9F illustrates low energy conformers of the peptide backbone of a diphosphorylated analogue of peptide τ 224-240 [SEQ ID NO: 11]. Low dielectric constant was used to mimic solvent effects (4.5 for TFE). Unconstrained simulated

annealing calculations were performed as described herein. The structures were overlaid automatically based on the similarity of all backbone atoms.

Fig. 10 demonstrates the specificity of anti-phosphopeptide antisera to unphosphorylated [SEQ ID NO: 8] and Thr231, Ser235 double phosphorylated [SEQ ID NO: 11] τ 224-240 peptides as well as normal τ and PHF- τ proteins. The double phosphorylated [SEQ ID NO: 11] τ 224-240 peptide was co-linearly synthesized with a turn-inducing spacer (Ala-Gly-Ala) and sequence 31D [SEQ ID NO: 25], and C3H mice were immunized with the chimeric peptide. After booster immunization, the anti-phosphopeptide mouse sera were analyzed for recognition of normal τ and its fragment as well PHF- τ and its fragment.

Fig. 11A illustrates binding of α - ω diamines and polyamines to the Thr231 monophosphorylated analog [SEQ ID NO: 9], to the Ser235 monophosphorylated analog [SEQ ID NO: 10] and to the Thr231, Ser235 double phosphorylated analog [SEQ ID NO: 11] of the τ 224-240 peptide. A modified ELISA protocol was used, in which the reduction of phosphorylation site-specific anti-PHF- τ antibody recognition was measured after the phosphopeptides were mixed with the various diamines and polyamines. The assay for the Thr231 monophosphorylated peptide [SEQ ID NO: 9] was developed with monoclonal antibody PHF-6 (specific for phosphorylated Thr231), for the Ser235 monophosphorylated peptide [SEQ ID NO: 10] with monoclonal antibody TG4 (specific for phosphorylated Ser235) and for the Thr231, Ser235 double phosphorylated peptide [SEQ ID NO: 11] with monoclonal antibody MC5 (specific for double phosphorylated Thr231 and Ser325).

Fig. 11B illustrates binding of α - ω diamines and polyamines to PHF- τ protein and to the Thr231, Ser235 double phosphorylated τ 224-240 peptide [SEQ ID NO: 11]. A modified ELISA assay was used, as described in Fig. 11A. The assay was developed with monoclonal antibody PHF-6 (specific for phosphorylated Thr231), with monoclonal antibody TG4 (specific for phosphorylated Ser235) and with monoclonal antibody MC5 (specific for double phosphorylated Thr231 and Ser325).

Detailed Description of the Invention

The present invention provides multiphosphorylated peptides useful in generating antibodies specific for the tau protein of the paired helical filaments found in the brain of patients with Alzheimer's disease. The inventors have found that
5 antibodies specific for Alzheimer's disease-originated tau protein seem to recognize a reverse-turn conformation between the phosphate sites. The multiphosphorylated peptides are able to bind multivalent compounds. It is this observation which permitted the inventors to find that the multiphosphorylated peptides and antibodies generated are useful diagnostically and/or therapeutically, as well as for drug design
10 for Alzheimer's disease.

I. Tau (τ) Peptide Fragments

The multiphosphorylated peptides of the invention are desirably derived from a fragment of the τ protein of the paired helical filaments which are present in the brains of Alzheimer's patients.

15 The τ protein may be obtained from a variety of sources, include adult-autopsy tissue, fetal sources, or biopsy-derived tissue, or may be synthesized using techniques known to those of skill in the art. All references to τ amino acid residues follow the numbering scheme provided in Goedert et al, Neuron, 3:519-526 (1989), which is incorporated by reference herein.

20 Desirably, the τ protein fragment which may serve as a template for constructing the peptides of the invention is between about 4 and about 20 amino acids in length, more preferably about 15 to about 20 amino acids in length. Exemplary τ protein fragments include the fragment spanning about amino acid residue 207 to about 222 of τ , about amino acid residue 224 to about 240 of τ , and
25 the fragment spanning about amino acid residues 390 to about 408 of τ . The sequences of these fragments are illustrated in Table 1 below. The present invention is not limited to these fragments. One of skill in the art can readily select other appropriate fragments given the guidance provided herein and the knowledge in the art.

According to the invention, a desired τ protein fragment is modified to contain, or synthesized to contain, at least two phosphorylated amino acids. In a preferred embodiment, the amino acids are serines or threonines, or a combination thereof. In a particularly preferred embodiment, the serine(s) and/or threonine(s) are separated by one to up to six amino acids, i.e., they may be immediately adjacent or separated by as many as six amino acids. For instance, exemplary phosphorylated peptides of the invention, which are derived from amino acids 207-222 of τ [SEQ ID NO: 1], include: GSRSR(T)P(S)LPTPTRE [SEQ ID NO: 5], GSRSR(T)PSLP(T)PPTRE [SEQ ID NO: 6], and GSRSRTP(S)LP(T)PPTRE [SEQ ID NO: 7], where the parentheses () indicate a phosphorylated amino acid. Another exemplary phosphorylated peptide of the invention includes KKVAVVR(T)PPK(S)PSSAK [SEQ ID NO: 11], which is derived from amino acids 224-240 of τ [SEQ ID NO: 8]. Still other exemplary phosphorylated peptides of the invention include AEIVYK(S)PVV(S)GDTSPRHL [SEQ ID NO: 17], AEIVYK(S)PVVSGD(T)SPRHL [SEQ ID NO: 18], AEIVYK(S)PVVSGDT(S)PRHL [SEQ ID NO: 19], AEIVYKSPVV(S)GD(T)SPRHL [SEQ ID NO: 20], AEIVYKSPVV(S)GDT(S)PRHL [SEQ ID NO: 21], and AEIVYKSPVVSGD(T)(S)PRHL [SEQ ID NO: 22], all of which are derived from amino acids 390-408 of τ [SEQ ID NO: 12]. Other suitable phosphorylated peptides of the invention, including those with more than two phosphorylated amino acids may be readily prepared by one of skill in the art based on these and other τ peptide fragments.

III. Production/Synthetic Peptides

Where desired, the τ protein fragments and the phosphorylated peptides of the invention are produced using synthetic techniques. For example, the peptides may be generated using a commercially available automatic synthesizer according to standard procedures. In this manner, phosphoserine or phosphothreonine residues may be incorporated in the course of synthesis.

Alternatively, other standard techniques may be utilized. See, e.g., Merrifield, J. Amer. Chem. Soc., 85:2149-2154 (1963).

Although less desired, in view of the size of the τ peptide fragments and the phosphorylated peptides of the invention, these fragments and peptides may be prepared by known recombinant DNA techniques by cloning and expressing, within a host microorganism or other cell, a DNA fragment carrying a coding sequence for a multiphosphorylated peptide, antibody or antibody fragment of the invention. Suitably, the expression system is selected from among those which are capable of adding the required number of phosphate groups, e.g., insect cells infected with baculovirus. Coding sequences for the peptides (or antibodies) of the invention can be prepared synthetically or can be derived from viral RNA by known techniques, or from available cDNA-containing plasmids. Thus, the invention also encompasses nucleic acid sequences encoding the peptides, antibodies and antibody fragments, of the invention. These nucleic acid sequences are useful not only for recombinant production methods, but may themselves be used in the diagnostic and therapeutic methods described herein.

Systems for cloning and expressing the phosphorylated peptides of the invention in various cells, including, for example, bacterial, mammalian, yeast and insect cells, and suitable vectors may be readily selected from among known and available from private and public laboratories and depositories and from commercial vendors. Currently, the most preferred host is a mammalian cell such as Chinese Hamster ovary cells (CHO) or COS-1 cells. These hosts may be used in connection with poxvirus vectors, such as vaccinia or swinepox. The selection of other suitable host cells and methods for transformation, culture, amplification, screening and product production and purification can be performed by one of skill in the art by reference to known techniques. See, e.g., Gething and Sambrook, Nature, 293:620-625 (1981). The antibodies of the invention and fragments thereof are also amenable to recombinant production techniques, such as those described herein. Another preferred system includes the baculovirus expression system and vectors. Bacterial expression may also be desired.

When produced by conventional recombinant means, the phosphorylated peptides of the invention may be isolated either from the cellular contents by conventional lysis techniques or from cell medium by conventional methods, such as chromatography. See, e.g., Sambrook et al., Molecular Cloning. A Laboratory Manual, 2d Edit., Cold Spring Harbor Laboratory, New York (1989).

IV. Production of Antibodies/Inhibitors

The phosphorylated peptides of the invention are useful in generating both polyclonal and monoclonal antibodies or for testing the binding of other bivalent compounds. Advantageously, these antibodies are specific for the phosphorylated peptides from which they are generated, i.e., the antibodies do not bind to normal τ fragments or other normal proteins.

Specific antisera (polyclonal antibodies) may be generated using known techniques. See, Sambrook, cited above, Chapter 18, generally, incorporated by reference. Similarly, monoclonal antibodies of the invention may be produced by conventional methods, including the Kohler and Milstein hybridoma technique, recombinant techniques, such as described by Huse et al, Science, 246:1275-1281 (1988), or any other techniques known to the art.

The invention further encompasses functional fragments of the antibodies of the invention, including, Fab, F_v , and $F(ab')_2$ fragments, synthetic molecules containing the binding site of the antibodies of the invention, and the complementarity determining regions (CDRs) thereof. Further, these functional fragments may be used in the production of recombinant antibodies, including bifunctional antibodies, chimeric antibodies, and humanized antibodies, which preferably retain the antigen binding specificity of the antibodies of the invention. Such recombinant antibodies may be constructed and produced according to known techniques [see, e.g., S. D. Gillies et al, J. Immunol. Meth., 125:191-202 (1989); and G. E. Mark and E. A. Pladlan, "Humanization of Monoclonal Antibodies", The Handbook of Experimental Pharmacology, Vol. 113, Chapter 4, pp. 105-133, Springer-Verlag (June, 1994)]. These functional fragments and recombinant

antibodies may be used for a variety of purposes, including any of those described herein for the antibodies of the invention.

In general, polyclonal antisera, monoclonal antibodies and other antibodies which bind to a phosphorylated peptide as the antigen (Ab1) are useful to
5 identify epitopes of τ , to separate τ from contaminants in living tissue (e.g., in chromatographic columns and the like), and in general as research tools and for the production of other types of antibodies described above. Anti-idiotypic antibodies (Ab2) are useful for binding τ and thus may be used in the treatment of Alzheimer's disease. Other uses as research tools and as components for separation of τ from
10 other contaminants of living tissue, for example, are also contemplated for these antibodies.

V. Diagnostic applications

Advantageously, the present invention provides reagents and methods useful in detecting and diagnosing Alzheimer's disease. Thus, the phosphorylated
15 peptides and antibodies of the invention may be useful as diagnostic reagents. These reagents may optionally be labelled using diagnostic labels, such as radioactive labels, colorimetric enzyme label systems and the like conventionally used in diagnostic or therapeutic methods. Alternatively, the – or C-terminus of a phosphorylated peptide of the invention may be tagged with a detectable label which can be recognized by a
20 specific antisera or another binding mechanism, e.g., biotin-streptavidin. The terminal labels include those detected by fluorimetric, colorimetric, etc., methods. The reagents may be used in diagnosis of Alzheimer's disease. For example, ELISA or other immunological methods can be used for the detection of hyperphosphorylated tau protein in cerebrospinal fluid (CSF) or blood; these methods make use of the
25 antibodies of the invention. The selection of the appropriate assay format and label system is within the skill of the art and may readily be selected by a skilled artisan.

Thus, the present invention provides methods for the detection of Alzheimer's disease. The methods involve contacting a selected mammalian tissue,

particularly brain tissue, or CSF, or serum, in a selected assay format based on the binding or hybridization of the reagent to the sample.

VI. Therapeutic Applications

Compositions and methods useful for the treatment of conditions associated with Alzheimer's disease are provided. The therapeutic compositions of the invention may be formulated to contain a phosphorylated peptide or antibody of the invention, or a fragment thereof. In one desirable embodiment, an antibody of the invention is used for passive immunotherapy. In another embodiment, the multiphosphorylated peptide is used as an immunogen for active immunotherapy. In still another embodiment, a compound (e.g., a bivalent reagent described below) identified through use of the multiphosphorylated peptide of the invention) is used for treatment of Alzheimer's disease. The therapeutic composition desirably contains 0.01 μ g to 10 mg peptide, protein, or reagent. These compositions may contain a pharmaceutically acceptable carrier. Suitable carriers are well known to those of skill in the art and include, for example, saline. Alternatively, such compositions may include conventional delivery systems into which protein of the invention is incorporated. Optionally, these compositions may contain other active ingredients, e.g., chemotherapeutics. The dose, timing and mode of administration of these compositions may be determined by one of skill in the art. Such factors as the age and condition of the patient, may indicate increasing or decreasing the dose, frequency or mode of administration of the therapeutic compositions of the invention. Generally, administration in a site-directed manner and is repeated as needed. Such therapy may be administered in conjunction with conventional therapies.

VII. Drug Design

The phosphorylated peptides of the present invention may also be used in the design, screening and development of simple chemical compounds, proteins, or complex biopolymers which have utility as diagnostic and/or therapeutic drugs for Alzheimer's disease.

As one example, a compound capable of bivalent binding to the multi-phosphorylated peptide or hyperphosphorylated tau protein of the invention and either preventing or enhancing its biological activity may be a useful drug component for the treatment or prevention of Alzheimer's disease. In particular, the ability of some

5 diamines and polyamines to selectively bind to the abnormally hyperphosphorylated tau peptides and proteins is demonstrated herein. Other suitable compounds may similarly bind concomitantly to two phosphate groups on the tau peptides or proteins described herein, i.e., phosphorylated tau peptides and proteins where the phosphate groups are separated by a distance of about 5 to 12Å. Such a compound may be

10 obtained using known techniques from a peptide library, an organic chemical library (including a library of diamines or polyamines), or derived from a variety of sources, including, for example, extracts of supernatants from culture of bioorganisms, extracts from organisms collected from natural sources, known chemical compounds, and mixtures thereof.

15 Utilizing the phosphorylated peptides or antibodies of the invention, conventional assays and techniques may be utilized for the screening and development of drugs capable of competitively binding to these peptides and proteins. An example of one suitable method involves the incubation of a test compound and the phosphorylated peptide or an antibody of the invention which may be immobilized on

20 a solid support. Alternatively, the incubation may be performed fully in solution (e.g., fluorescence polarization). Still other conventional methods of drug screening can involve employing a suitable computer program to determine compounds having similar or a complementary chemical structure to that of a phosphorylated peptide or antibody of the invention and screening those compounds for competitive binding.

25 Thus, through use of such methods, the present invention is anticipated to provide compounds capable of interacting with the phosphorylated peptides of the invention, and either enhancing or decreasing its biological activity, as desired. Such compounds are encompassed by this invention.

It should be understood that one of skill in the art may readily select the type of conventional screening method most desirable, as well as the reagent of this invention, e.g., the phosphorylated peptide, antibody or fragment thereof.

Desirably, these designed drugs, or drugs identified through these
5 screening assays, are specific for Alzheimer's disease and do not interact with normal proteins, including normal τ . Such specificity can be assessed using known techniques, including those illustrated in the examples below. For example, specificity of binding can be determined by comparing the binding of the identified compound (e.g., an inhibitor) to a multiphosphorylated peptide of the invention and to a single
10 phosphorylated version of the same amino acid stretch or negative control unphosphorylated or multi-phosphorylated sequences. Particularly suitable multiphosphorylated peptides useful for the drug design are GRSR(T)P(S)LPTPPTRE [SEQ ID NO: 5] and KKVAVVR(T)PPK(S)PSSAK [SEQ ID NO: 11]. Particularly suitable control sequences include
15 ADEV(S)A(S)LAKQGL (corresponding to amino acids 429-442 from the C-terminal end of τ , SEQ ID NO: 23) and multiphosphorylated sequences from normal proteins, such as those from neurofilaments (e.g., EEKGK(S)PVPK(S)PVEEKG, SEQ ID NO: 24).

These examples illustrate the preferred methods for preparing the
20 phosphorylated peptides and the antibodies of the invention. These examples are illustrative only and do not limit the scope of the invention.

Example 1 - Immunization with PHF

Highly purified PHF- τ preparations were prepared as described [V.M.-
Y. Lee et al, Science, 251:675-678 (1991)]. Immunization with PHF- τ and fusion with
25 mouse myeloma cells SP2/0-Ag14 followed previously published protocols (Lee et al, (1987)].

Immunization of mice with PHF τ yielded a number of mAbs that were tested for their antigen recognition by Western-blot and ELISA, as described in Example 2 below. The antigens included adult autopsy-derived τ , fetal τ , and PHF τ .

As controls, four existing mAbs were added to the mAb pool: AT10 [H.E. Mercken et al, EMBO J., 11:1593-1597 (1992)]; PHF-1 [S. G. Greenberg, et al, J. Biol. Chem., 267:564-569 (1992)], AT180 [M. Goedert et al, Biochem. J., 301:871-877 (1994)], and TG3 [Vincent et al, J. Cell. Biol., 132:413-425 (1996)]. PHF-1 and AT180 have
5 been reported to label fetal τ and PHF τ , but not autopsy-derived normal adult τ (Matsuo et al, 1994). In contrast, mAb AT10 labels only PHF- τ on Western blot (Matsuo et al, 1994) and TG3 labels predominantly PHF-tau [Vincent et al, 1996, cited above].

Example 2 - Western Blotting and Enzyme-linked immunosorbant assay (ELISA)

10 The epitopes of 12 monoclonal antibodies (mAb) raised against PHF (as described in Example 1) that recognized PHF- τ , but not normal τ on Western-blot and enzyme-linked immunosorbent assay (ELISA) were mapped. The epitope analysis, together with known binding sites of previously published and widely used mAbs, revealed Ser214, Thr231 and Ser396 as immunodominant phosphorylated
15 amino acids in PHF- τ . All mAbs recognizing these phosphorylated sites, with the exception of PHF-27, also labeled fetal τ and biopsy-originated τ . In contrast to previous findings, these data indicate that Ser214 can be phosphorylated in fetal τ .

A. ELISA

Direct ELISA was applied to look for regular or conformation-sensitive epitopes [E. Lang, et al, J. Immunol. Meth., 170:103-115 (1994); L. Otvos, Jr. and G. I. Szendrei, Enzyme-linked immunosorbent assay of peptides. In: Neuropeptide Protocols (Levine, B., and Williams, C., eds) Human Press, Totowa, pp. 269-275 (1996)]. Briefly, binding of 0.04-5 μ g amounts of peptide or
20 phosphopeptide antigens was tested with 1/50 to 1/3600 dilutions of mAbs on 96-well Nunc Immuno MaxiSorp plates. The antigens were dissolved in water or trifluoroethanol (TFE), and dried down to the plates at 37°C overnight. All subsequent steps were performed according to general ELISA protocols [J. W. Goding, Monoclonal Antibodies: Principles and Practice. Academic Press, Orlando (1986)]. Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin was
25

used as a secondary antibody at 1/1000 dilution. Color development was made with o-phenylenediamine, stopped with 1M aqueous H_3PO_4 , and absorption was measured at 450 nm. Purified autopsy-derived normal τ , and PHF- τ at 5 $\mu g/ml$ concentration were used as controls, and processed for ELISA as discussed above.

5 B. Western-blot

Western-blotting using autopsy-derived normal adult τ , fetal τ , PHF τ was performed as previously described [Matsuo et al, (1994), cited above]. Briefly, nitrocellulose replicas of gels containing electrophoretically separated τ samples were prepared from 10% sodium dodecyl sulfate-polyacrylamide gels and
10 probed with the primary antibodies. MAb binding was detected as previously described (Lee et al, 1991, cited above). Protein concentrations in the samples were determined using bicinchonic acid as a dye reagent with bovine serum albumin as the standard (Smith et al, 1985). The amount of protein loaded depends on the different τ preparations which varied from 1.5 to 12 μg . The protein concentration was adjusted
15 to obtain approximately equally strong immunoreactivity (if binding occurs at all) of various samples, based on binding of the same preparations to phosphorylation-independent antibody standards T14/46 (Matsuo et al, 1994, cited above). Each experiment was repeated at least three times.

Example 3 - Peptide Synthesis

20 In this study, highly purified synthetic peptides, as well as single and double phosphorylated analogues, were used to identify the binding sites of four of the frequently used antibodies, AT180 (Goedert et al., 1994, cited above), PHF-1 (Greenberg et., 1992, cited above), and AT10 [Mercken et al, 1992, cited above] and TG3 [Vincent et al, 1996, cited above], and of 12 mAbs to full-sized PHF proteins,
25 described herein. Since phosphate transfer is a common side-reaction during phosphopeptide synthesis and storage, the integrity of the antigens was continuously verified.

Peptides were synthesized on Milligen 9050 and Rainin PS3 automatic synthesizers using 9-fluorenylmethoxycarbonyl amino acids according to standard

procedures [G.B. Fields and R. L. Noble, Int. J. Pept. Protein Res., 35: 161-214 (1990)]. Phosphoserine or phosphothreonine residues were incorporated as Fmoc-Ser/Thr(PO₃HBzl)-OH [T. Wakamiya et al, Chem. Lett., 1099-1102 (1994)] purchased from Novabiochem, Ltd. Peptides and phosphopeptides were detached
5 from the solid support with trifluoroacetic acid (TFA) and they were purified by reversed-phase high performance liquid chromatography (RP-HPLC) using an aqueous acetonitrile gradient elution system containing 0.1% TFA as an ion pairing reagent. The integrity of the peptides and phosphopeptides was verified by mass spectroscopy. Table 1 lists the synthetic peptides. An asterisk indicates a
10 phosphorylated amino acid. These peptides are designated by SEQ ID NOS: 1-22, respectively. In other words, τ 207-222 is SEQ ID NO: 1 and the doubly phosphorylated τ 390-408 (403P, 404P) is SEQ ID NO: 22.

Table 1

Synthetic peptides and their characterization.

	Peptides	Sequence [min]	Retention time	Calculated mass [M+H] ⁺	Observed mass [M+H] ⁺
5	τ207-222	GSRSRTPSLPTPTRE	23.8	1739	1739
	212P	T*	23.6	1819	1821
	214P	S*	23.6	1819	1819
10	217P	T*	24.2	1819	1820
	212P,214P	T*S*	23.1	1899	1898
	212P,217P	T* T*	23.9	1899	1900
	214P,217P	S* T*	23.6	1899	1898
15	τ224-240	KKVAVVRTPPKSPSSAK	19.7	1780	1780
	231P	T*	20.0	1860	1860
	235P	S*	19.8	1860	1860
	231P,235P	T* S*	19.9	1940	1940
20	τ390-408	AEIVYKSPVVSGDTPRHL	25.5	2055	2055
	396P	S*	24.1	2135	2134
	400P	S*	24.9	2135	2134
	403P	T*	25.1	2135	2136
	404P	S*	25.3	2135	2136
	396P,400P	S* S*	24.5	2215	2214
	396P,403P	S* T*	26.6	2215	2217
25	396P,404P	S* S*	24.3	2215	2214
	400P,403P	S* T*	25.3	2215	2215
	400P,404P	S* S*	27.3	2215	2214
	403P,404P	T*S*	32.0	2215	2214

The new mAbs detect PHF-τ but not normal τ. Epitope analysis

30 revealed immunodominant phosphoserine and phosphothreonine residues in PHF. One of the newly developed mAbs, PHF-27, labels only PHF-τ, insignificantly labels fetal τ, and does not label biopsy-originated τ. What additional factors the PHF-specific mAbs (e.g. PHF-27) would detect was studied by studying the conformation of the epitopes.

Example 4 - Antibody Binding

The antibodies prepared as described in Example 3 above were tested for their binding to three synthetic peptide families, which were prepared as described below. All three peptide groups contained unphosphorylated, monophosphorylated
5 and diphosphorylated peptides.

Among the newly developed mAbs, only mAb PHF-27 binds specifically only to PHF- τ and not fetal, autopsy or biopsy-derived τ . These results indicate that like mAb AT10, mAb PHF-27 is a PHF-specific antibody.

As show herein, the peptide recognition of mAb PHF-27 was markedly
10 increased when both the primary site Thr231 and the subsite Ser235 were phosphorylated. This conformation-sensitive ELISA, supported by circular dichroism measurements and molecular modeling of the phosphorylated peptide epitope (see examples 5 and 6 below), indicated that while the other antibodies recognized just the phosphorylated immunodominant amino acids, mAb PHF-27 recognized both the
15 presence of the phosphate group and the conformation of the multiphosphorylated antigen. These data suggest that it is possible to develop true PHF-specific mAbs, but due to the limited epitope repertoire of the full protein, it is more beneficial to use designed, multiphosphorylated peptide immunogens, such as those of the invention.

Of the new 12 mAbs tested, five recognized phosphoamino acids in
20 one of the three peptide families above. In fact, three major immunodominant residues could be identified, i.e., phosphorylated Ser214, Thr231, and Ser396 (Table 2).

Table 2

Immunodominant phosphoamino acids in PHF- τ .

	Monoclonal antibody	Recognized primary phosphate site	Secondary phosphate site
5	New antibodies		
	PHF-20	Ser214	Thr217
	PHF-6	Thr231	
	PHF-27	Thr231	Ser235
10	PHF-13	Ser396	
	PHF-47	Ser396	
	Existing antibodies used in this study		
	AT10	Thr212 and Ser214 together	
	AT180	Thr231	
15	Literature data on the same sites ¹ :		
	M4	Thr231 ²	
	PHF-1	Ser396	Ser404 ³
	AD-2	Ser396	Ser404

- ¹ This list contains only mAbs generated by immunizing with PHF- τ protein.
 20 ² M. Hasegawa et al., *FEBS Lett.*, **384**:25-30 (1996).
³ Otvos et al, 1994.
⁴ Buee-Scherrer et al, *Mol. Brain Res.*, **39**:79-88 (1996).

Mass spectrometry indicate Thr231 and Ser235, two phosphorylated residues of PHF- τ in the AT180 epitope region: [Morishima-Kawashima et al., *J. Biol. Chem.*, **270**:823-829 (1995)]. The τ 224-240 peptide [SEQ ID NO: 8], the two
 25 monophosphorylated analogues (231P and 235P) [SEQ ID NOS: 9 and 10,

respectively] and the diphosphorylated peptide (231P,235P) [SEQ ID NO: 11] were synthesized in a way which places the phosphoamino acids in the middle of the molecule (Table 1). These peptides were made long enough to cover the entire epitope and possess some secondary structure, but short enough to allow preparation with the highest possible purity and not to have more than two conformational elements. MAb AT180 did not bind to unphosphorylated peptide τ 224-240 [SEQ ID NO: 8] or to the phosphorylated version 235P [SEQ ID NO: 10], but did bind to the same peptide phosphorylated at Thr231 [SEQ ID NO: 9] and to the diphosphorylated peptide 231P, 235P [SEQ ID NO: 11] (Table 2). Once Thr231 was phosphorylated, the antigen recognition of mAb AT180 was not increased after phosphorylation of Ser235. MAb AT180 bound to the Thr231 phosphorylated peptide very strongly. As little as 40ng phosphopeptide could be clearly detected at antibody dilution of 1:3600.

The antigen recognition pattern of new antibody mAb PHF-6 was completely identical to that of mAb AT180. MAb PHF-6 did not recognize the unphosphorylated [SEQ ID NO: 8], or Ser235 phosphorylated [SEQ ID NO: 10] τ 224-240 peptide, and recognized the Thr231 phosphorylated [SEQ ID NO: 9], and diphosphorylated peptides [SEQ ID NO: 11] equally well (Fig. 3). In contrast to these antibodies, the antigen recognition of mAb PHF-27 showed a different pattern. This mAb did not bind to the unphosphorylated τ 224-240 peptide [SEQ ID NO: 8], as expected. Like mAb PHF-6 and AT180, mAb PHF-27 recognized phosphorylated Thr231 [SEQ ID NO: 9] as a main site and did not recognize phosphorylated Ser235 [SEQ ID NO: 10]. When both Thr231 and Ser235 were phosphorylated [SEQ ID NO: 11], however, the antigen recognition was markedly increased, especially at low antigen amounts (Fig. 4).

MAbs PHF-47 and PHF-13, just like PHF-1, did not recognize unphosphorylated peptide τ 390-408 [SEQ ID NO: 12], or the same peptide phosphorylated at Ser400 [SEQ ID NO: 14], Thr403 [SEQ ID NO: 15] or Ser404 [SEQ ID NO: 16], but recognized the Ser396 phosphorylated peptide [SEQ ID NO: 13], and all diphosphorylated peptides containing phosphorylated Ser396 [SEQ ID NOS: 17-19]. None of the diphosphorylated peptides lacking phosphate group on

Ser396 [SEQ ID NOS: 20-22] were recognized by these mAbs (Table 2). MAb PHF-20.6 (subclone 6 of antibody 20) did not bind to the unphosphorylated peptide τ 207-222 [SEQ ID NO: 1], or to the peptide phosphorylated at Thr212 (212P) [SEQ ID NO: 2]. Weak binding at high peptide loads was observed for
5 monophosphorylated peptide 217P [SEQ ID NO: 4], and diphosphorylated peptide 212P, 217P [SEQ ID NO: 6]. The antibody binding was strong to Ser214 monophosphorylated peptide (214P) [SEQ ID NO: 3] (Table 2) but was not significantly increased after incorporation of a second phosphate group to Thr212 [SEQ ID NO: 5] or Thr 217 [SEQ ID NO: 7]. Thus, detailed epitope analysis
10 indicated that mAb PHF-20.6 recognized phosphorylated Ser214 as a main site and very slightly recognized the surrounding subsites.

After identifying the immunodominant phosphoamino acids in PHF- τ , the three peptide families were used to identify the epitope of mAb AT10. As earlier attempts with monophosphorylated peptides and simple mutated proteins failed to
15 identify the antigen binding site for this mAb, diphosphorylated peptides were concentrated upon. Indeed, no antibody binding was detected to any of the 12 unphosphorylated or monophosphorylated synthetic peptides, but the mAb bound to the antigens when both Thr212 and Ser214 were simultaneously phosphorylated (Fig. 5) [SEQ ID NO: 5]. Interestingly, this mAb failed to recognize the other two
20 diphosphorylated versions of the same peptide, namely those that carried phosphate groups on Thr212 and Thr217 [SEQ ID NO: 6], or Ser214 and Thr217 [SEQ ID NO: 7]. These data, together with those obtained for mAbs PHF-27 clearly indicate that PHF specific epitope include doubly phosphorylated Thr212/Ser214 [SEQ ID NO: 5] and Thr231/Ser235 [SEQ ID NO: 11].

25 Which factors were recognized by mAb PHF-27 and TG3 recognized and mAbs PHF-6 and not by AT180 were then determined. All four antibodies saw phosphorylated Thr231 and did not see individually phosphorylated Ser235. To make this determination, the peptides that were recognized by the antibodies [i.e. 231P [SEQ ID NO: 9] and 231P,235P [SEQ ID NO: 11] (marked PP in the figure)] were
30 plated for the ELISA from water and also from TFE. With mAbs PHF-6 and AT180

the antigen recognition was increased for both peptides when the conformation of the phosphopeptides was stabilized in TFE (Fig. 6A). This indicates that these antibodies recognized a peptide conformation that was not dominant in water but could be generated in TFE. When the antigen recognition of mAb PHF-27 was studied, the recognition was dramatically increased for the monophosphorylated peptide plated from TFE, but not for the already strong binder diphosphorylated peptide (Fig. 6B). This finding indicated that antigen recognition by the antibody was stronger toward the diphosphorylated peptide in aqueous solutions because mAb PHF-27 saw both the presence of the phosphate group at Thr231, and the conformation of the diphosphorylated peptide that it assumed in water. MAb PHF-6 and AT180 saw the phosphorylated Thr231 residue, but did not see the conformation of the diphosphorylated peptide in water. Mab PHF-27 stands out in Western-blot as well. The recognition of the 12 new mAbs was analyzed against normal τ , fetal τ , biopsy-derived τ and PHF- τ . None of the antibodies recognized normal τ , and all bound to PHF- τ . Nevertheless, all (including AT180 and PHF-1) but PHF-27 also bound to fetal τ and biopsy-derived τ . These results indicate that mAb PHF-27 is the a "true" PHF-specific antibody. Based on the conformation-sensitive ELISA assay, it is tempting to speculate that mAb PHF-27 recognized a specific multiphosphorylated peptide secondary structure. It is worth noting that similarly to mAb PHF27, the antigen recognition of AT10 did not increase when the diphosphorylated peptide antigen was plated from TFE, just the "pro-zone" binding was shifted to lower antigen amounts (data not shown). Mab PHF27 appeared to be weaker than the other anti-PHF antibodies [i.e. weaker peptide (compare Figs. 3 and 4) or protein (Fig. 1) recognition with the same antibody dilution compared, for example to mAb PHF-6 that recognizes the same primary site].

Monoclonal antibodies TG3 [Vincent et al., 1996 cited above] and AT180 [Goedert et al., 1994, cited above] recognize phosphorylated Thr231. To test the hypothesis that TG3 and AT180 may recognize different conformations of the protein, the binding of the four 224-240 synthetic peptides to these antibodies was analyzed by using our conformation-sensitive ELISA protocol (as described above).

Both the unphosphorylated [SEQ ID NO: 8] and the Ser235 phosphorylated [SEQ ID NO: 10] peptides were devoid of either TG3 or AT180 reactivity regardless of which solvent was used to apply the peptides to the plate (Fig. 7). AT180 was found to be nearly equally reactive with both the monophosphorylated Thr231 peptide [SEQ ID NO: 9] and the diphosphorylated peptide [SEQ ID NO: 11] regardless of the solvent conditions. Analysis of TG3 gave quite different results (Fig. 7). TG3 reactivity with the Thr231 phosphorylated peptide [SEQ ID NO: 9] was substantially increased after drydown from TFE, suggesting that stabilization of the local peptide conformation plays an important role in determining TG3 reactivity. Also, unlike AT180, TG3 was virtually unable to bind the diphosphorylated peptide [SEQ ID NO: 11] after drydown from water. The binding of mAb TG3 to the diphosphorylated peptide [SEQ ID NO: 11] was restored however, when the peptide was applied from TFE (Fig. 7), clearly showing that a certain conformation of the diphosphorylated peptide is needed for TG3 recognition. Based on the CD analysis performed in water and in TFE, this conformation is a reverse-turn.

In summary, by using a panel of monoclonal antibodies directed to PHF- τ , the presence of a phosphate group on Ser214 of fetal τ [SEQ ID NO: 3], and the concomitant presence of phosphates on Thr231 and Ser235 of PHF- τ [SEQ ID NO: 11], were positively identified. MAb 20 labels fetal τ but not normal τ on Western-blot indicating that Ser214 carries a phosphate in immature brain. On the other hand, both Thr212 and Ser214 [SEQ ID NO: 5] needed to be phosphorylated for any recognition by mAb AT10 and both Thr231 and Ser235 [SEQ ID NO: 11] needed to be phosphorylated for full recognition by mAb PHF-27. MAb PHF-27 did not recognize normal τ or biopsy-derived τ and very insignificantly fetal τ . MAb PHF-27 appears to be the a true PHF-specific antibody, and useful as a reagent for the development of AD-specific diagnostic markers. The binding of PHF-27 to PHF- τ in normal body fluids remains to be determined, albeit the low dilution of this antibody needed for ELISA and Western-blot indicates that PHF-27 may not be the final solution for this purpose. Based on the speculation of Goedert (cited above) Ser235 needs to be phosphorylated before Thr231 can be phosphorylated. τ can be

phosphorylated at Thr231 by glycogen synthase kinase 3 only if Ser235 is already phosphorylated by mitogen activated protein (MAP) kinase. If this is indeed the reality, these results suggest that Ser loses its phosphate group during the brain development, but regains the phosphate during the development of AD. This

5 explanation is consistent with both the current dynamic multiple kinase-phosphatase equilibrium theory that interprets normal microtubule assembly or abnormal PHF aggregation (Garver et al., 1996; Singh et al., 1996), and with the different sensitivity of these sites for phosphatase actions. While phosphorylated Ser235 is a preferential substrate site for protein phosphatase 2B [Gong et al, J. Neurochem., 62:803-806

10 (1994)], phosphorylated Thr231 is an unusually resistant residue to dephosphorylation [Hasegawa et al, J. Biol. Chem., 267:17047-17054 (1992)]. While Thr231 and Ser235 can be phosphorylated by a number of kinases *in vitro* [Morishima-Kawashima et al, (1995), cited above], Thr212 and Ser214 are substrates for specific kinases. Thr212 can only be phosphorylated by MAP kinase [Drewes et al, (1992), cited

15 above] and brain kinase [Biernat et al, (1993), cited above], and Ser214 can only be phosphorylated by cyclic AMP-dependent protein kinase (cAMP-PK) [Scott et al, (1993), cited above]. Nevertheless, this site is a major target for cAMP-PK: 50% of phosphate incorporated into τ by cAMP-PK is on Ser214 [Letersky et al, (1996), cited above]. MAP kinase transforms τ to an AD-like immunological state and

20 phosphorylation of Ser214 is one of the major factors responsible for decreasing the ability of τ to nucleate microtubules [Brandt et al, (1994), cited above]. By requiring phosphate groups on both Thr212 and Ser214 for binding, mAb AT10 appeared to fortunately carry recognition sites for both AD features: lack of microtubule-binding and PHF-like immunoreactivity.

25 Example 5 - Circular dichroism (CD)

Full size normal human τ possesses only a negligible fraction of periodic (α -helix or β -pleated sheet) conformations in solution [D. W. Cleveland et al, J. Mol. Biol., 116:227-247 (1977)] and this is independent of whether τ is from the

brain or expressed bacterially. The secondary structure of the 22 synthetic peptides of Table 1 was studied.

CD spectra were taken on a Jasco J720 instrument at room temperature in a 0.2 mm path length cell. Double distilled water and spectroscopy grade trifluoroethanol were used as solvents. The peptide concentration was about 0.5 mg/ml, determined each time by quantitative RP-HPLC [G. I. Szendrei, et al, Eur. J. Biochem., 226:917-924 (1994)]. Curve smoothing was accomplished by the algorithm provided by JASCO. Mean residue ellipticity ($[\Theta]_{MR}$) is expressed in degrees/dmole by using a mean residue weight of 110. Because the secondary structures of the peptides (especially phosphopeptides) provided by the current computer-assisted curve analyzing algorithms show a high error rate, the CD spectra evaluations were based on comparison with known peptide conformations [R. W. Woody, et al, Circular dichroism of peptides. In: The Peptides (Hruby, V.J., ed.) Academic Press, Orlando, pp. 15-114 (1985); L. Otvos, L., Use of circular dichroism to determine secondary structure of neuropeptides. In: Neuropeptide Protocols (Irvine, B., and Williams, C., eds) Human Press, Totowa, pp. 153-161 (1996)].

None of the 22 synthetic peptides shown in Table 1 exhibited any β -sheet component by CD. Instead, these peptides exhibited CD spectra indicative of various equilibriums of unordered and turn conformers.

To explain the specificity of mAb PHF-27 and TG3 for PHF- τ , the conformation of the τ 224-240 peptides was studied in water and TFE. In water all four of the τ 224-240 peptides exhibited type U CD spectra, characteristic of peptides without conformational preferences (Woody, cited above). The lack of the small positive band around 220 nm indicated the presence of some ordered structures (Fig. 8A). Based on the CD of the peptides in TFE, this residual ordered structure was likely to be some turn conformation. The peptides exhibited type C CD spectra in TFE (Fig. 8B), characteristic of type I β turns [F. A. Smith and L. G. Pease, CRC Crit. Rev. Biochem., 8:315-399 (1980)] or mixtures of β -turns with dominant type I character [A. Perczel et al., Int. J. Pept. Protein Res., 41:223-236 (1993)]. Among the four peptides, the turn character was far stronger for the diphosphorylated

peptide, than for either of the unphosphorylated or the monophosphorylated variants. This in turn, may indicate that mAb PHF-27, as opposed to mAbs PHF 6 and AT1890, saw not only the presence of the phosphate group, but also the residual turn conformation of the diphosphorylated peptide in water. Antibody TG3 recognized the double phosphorylated peptide only when the antigen assumed turn structure. This may indicate that in PHF-tau, phosphorylated Thr231 and Ser235 define a reverse turn. This turn structure, specific for PHF-tau can be utilized in the design of inhibitors that would bind the abnormally multi-phosphorylated protein variant. To justify this assumption of the turn structure, simulated annealing calculations were run on the unphosphorylated, the Thr231 phosphorylated, and the diphosphorylated peptides. See Example 6.

Example 6 - Molecular modeling

Molecular modeling and conformational energy calculations were performed with the Quanta 4.1/CHARMm 22 software packages on a Silicon Graphics Indy R4600PC work station. All atom parameters were assigned automatically from the consistent valence force field library provided by the software. Dielectric constants (4.5 for TFE, and 78 for water) were employed in scaling electrostatic interactions [E. D. Getzoff et al, *Nature*, 306:287-290 (1983)]. To search the full conformational space which could be occupied by the molecules, a high-temperature molecular dynamics and simulated annealing protocol was implemented. The peptides were built up in type 1 β -turn structure, and the energy was minimized by the steepest descents method. Molecular dynamics were run between 0-900 K. The molecules were heated in 6120 steps by 0.001 ps increments, equilibrated for 6000 steps and the simulation was run for 6000 steps. From the structures generated during the simulation, four low energy structures from various regions of the trajectory were randomly selected. The four structures were cooled to 300 K over 18 ps. Each structure was energy minimized as before the simulated annealing.

The modeling fully supported the spectroscopic and immunological findings. Low energy conformers in high dielectric constant environment were consistent with an increased population of turn structures of the diphosphorylated peptide (Fig. 9E) compared to the monophosphorylated (Fig. 9C) or the
5 unphosphorylated analogue (Figs. 9A). Low dielectric constant environment stabilized the turn of the two phosphopeptides (Figs. 9D and 9F).

In addition to the selective recognition of the diphosphorylated peptide in water by MAb PHF-27 and the selective recognition of a turn structure of the diphosphorylated peptide by Mab TG3, two conclusions can be drawn.

10 1) The binding of the studied anti-PHF antibodies to the phosphopeptides is strongly dependent upon the length and/or the purity of the antigens as well as the dilution of the antibody preparation. Mabs PHF-20, PHF-47 and PHF-1 recognized phosphorylated amino acids other than the main sites (Ser214 for PHF-20 and Ser396 for PHF-1) in antigen preparations where the antigens were
15 longer or less homogenous.

2) PHF- τ contains immunodominant phosphoserine and phosphothreonine residues in neighboring sites. The three most characteristic single sites are Ser214, Thr231 and Ser396 with participation of nearby phosphoaminoacids (Thr212, Thr217, Ser235 and Ser404). The mAbs against these phosphoamino acids
20 were always dominant and the mAbs showed the same specificity. In turn, this suggests that the antibody repertoire that can be produced by using the full protein as immunogen is limited. Synthetic phosphopeptides, which are much shorter than the protein, and contain designed phosphoserines or phosphothreonines, display and present each of the phosphoamino acids much more efficiently than the long,
25 heterogeneously phosphorylated protein does. Antibodies against designed phosphopeptides offer considerably increased variety and specificity to hidden linear or conformational epitopes that may be singly or multiply phosphorylated in PHF- τ , but not in normal τ , fetal τ or biopsy-originated τ .

Example 7 - Generation of anti-phosphopeptide monoclonal antibodies specific for PHF- τ .

To obtain sensitive and selective antibodies to PHF- τ , a chimeric peptide construct was prepared, in which the immunodominant diphosphorylated τ 224-240 peptide [SEQ ID NO: 11] was co-linearly synthesized with a turn-inducing spacer (Ala-Gly-Ala) and the sequence 31D [SEQ ID NO: 25], which is a T-helper cell epitope of the rabies virus nucleoprotein [Ertl et al., *J. Virol.*, 63: 2885-2892, (1989)] in a T - B epitope orientation. Twenty μ g of the chimeric construct was mixed with 50% complete Freund's adjuvant and was injected into the hind legs of C3H mice. Fourteen days later, the mice received a booster immunization of 20 μ g of the chimeric peptide in 50% incomplete Freund's adjuvant. Ten days after the booster immunization, the resulting antisera were analyzed for antigen specificity (Fig. 10). The sera exhibited the same diphosphorylated peptide and PHF- τ protein specificity as mAb PHF-27 on ELISA, but with considerably increased sensitivity.

Example 8 - Binding of the phosphopeptides and the PHF- τ protein to α - ω diamines and polyamines.

The feasibility of the hypothesis was tested, i.e. that the abnormally hyperphosphorylated peptide and protein variants assume a structure that places the phosphate groups around a reverse-turn, and suitable molecules, which will selectively bind to the multiphosphorylated sequences, can be identified. Diamines can simultaneously interact with the two phosphate groups *via* ionic forces. To this end, the peptide and protein solutions were mixed with α - ω diamines and polyamines and applied to the ELISA plate. To assess binding, a modified ELISA protocol was used, in which the reduction of phosphorylation site-specific anti-PHF- τ antibody recognition was measured after the phosphopeptides were mixed with the various diamines and polyamines. Three monoclonal antibodies were used, each specific to a given phosphate form of the antigens. The assay for the Thr231 monophosphorylated peptide [SEQ ID NO: 9] was developed with monoclonal antibody PHF-6 (specific for phosphorylated Thr231), for the Ser235 monophosphorylated peptide [SEQ ID

NO: 10] with monoclonal antibody TG4 (specific for phosphorylated Ser235) and for the Thr231, Ser235 double phosphorylated peptide [SEQ ID NO: 11] with monoclonal antibody MC5 (specific for double phosphorylated Thr231 and Ser325). Monoclonal antibodies TG4 and MC5 were provided by Dr. Peter Davies, Albert
5 Einstein College of Medicine, Bronx, New York. The amino groups of the diamines were separated by 2-12 methylene groups, or by saturated or unsaturated ring systems. Polyamines included spermine and spermidine. Three diamines, 1,8-diamino-octane (#6), 1,10-diamino-decane (#7) and 1,12-diamino-dodecane (#8) bound to all three peptides (unspecific binding) (Fig. 11A). A number of diamines and
10 polyamines bound only to the double phosphorylated peptide, most strongly the polyamines spermine (#16) and spermidine (#17) (Fig. 11A).

In the next step whether some of the diamines were able to bind the PHF- τ protein by using the same modified ELISA protocol was investigated. This assay is a complex assay, and therefore variable results were obtained. Nevertheless
15 three amines (1,8-diamino-octane, spermine and spermidine) that bound to the peptides strongly, reduced the O.D. reading more than 0.1 (Fig. 11B), which value is generally accepted in ELISA as the sign of clearly positive binding. Significantly, the distance between the amino groups of the best binder diamines or between the amino groups at one side of the polyamines is around 10 Å, very close to the distance
20 between the phosphate groups of our computer models of the double phosphorylated 224-240 τ peptide [SEQ ID NO: 11], when the phosphopeptide assumes a turn conformation. Based on these findings it is possible to identify simple compounds or biopolymers or their fragments that would selectively bind to the nearby phosphate groups of abnormally hyperphosphorylated PHF- τ protein and perhaps will be able to
25 eliminate PHF- τ if its is once formed.

Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modifications and alterations to the compositions and processes of the present invention are believed to be encompassed in the scope of the claims
30 appended hereto.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Wistar Institute of Anatomy & Biology
Trustees of the University of Pennsylvania
Otvos, Laszlo
Hoffmann, Ralf
Lee, Virginia

(ii) TITLE OF INVENTION: Diagnostic and Therapeutic
Reagents for Alzheimer's Disease

(iii) NUMBER OF SEQUENCES: 25

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Howson and Howson
(B) STREET: Spring House Corporate Cntr., PO Box 457
(C) CITY: Spring House
(D) STATE: PA
(E) COUNTRY: USA
(F) ZIP: 19477

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: WO
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 60/031,169
(B) FILING DATE: 19-NOV-1996

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Kodroff, Cathy A.
(B) REGISTRATION NUMBER: 33,980
(C) REFERENCE/DOCKET NUMBER: WST77APCT

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 215-540-9200

(B) TELEFAX: 215-540-5818

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly	Ser	Arg	Ser	Arg	Thr	Pro	Ser	Leu	Pro	Thr	Pro	Pro	Thr	Arg	Glu
1				5				10						15	

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 6

(D) OTHER INFORMATION: /product= "OTHER"
/note= "phosphorylated Thr"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Gly	Ser	Arg	Ser	Arg	Thr	Pro	Ser	Leu	Pro	Thr	Pro	Pro	Thr	Arg	Glu
1				5				10						15	

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 8
- (D) OTHER INFORMATION: /product= "OTHER"
/note= "phosphorylated Ser"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Gly Ser Arg Ser Arg Thr Pro Ser Leu Pro Thr Pro Pro Thr Arg Glu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 11
- (D) OTHER INFORMATION: /product= "OTHER"
/note= "phosphorylated Thr"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Gly Ser Arg Ser Arg Thr Pro Ser Leu Pro Thr Pro Pro Thr Arg Glu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 6
- (D) OTHER INFORMATION: /product= "OTHER"
/note= "phosphorylated Thr"

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 8
- (D) OTHER INFORMATION: /product= "OTHER"
/note= "phosphorylated Ser"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Gly Ser Arg Ser Arg Thr Pro Ser Leu Pro Thr Pro Pro Thr Arg Glu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 6
- (D) OTHER INFORMATION: /product= "OTHER"
/note= "phosphorylated Thr"

37

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 11

(D) OTHER INFORMATION: /product= "OTHER"

/note= "phosphorylated Thr"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Gly	Ser	Arg	Ser	Arg	Thr	Pro	Ser	Leu	Pro	Thr	Pro	Pro	Thr	Arg	Glu
1				5					10					15	

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 8

(D) OTHER INFORMATION: /product= "OTHER"

/note= "phosphorylated Ser"

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 11

(D) OTHER INFORMATION: /product= "OTHER"

/note= "phosphorylated Thr"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Gly	Ser	Arg	Ser	Arg	Thr	Pro	Ser	Leu	Pro	Thr	Pro	Pro	Thr	Arg	Glu
1				5					10					15	

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

38

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Lys Lys Val Ala Val Val Arg Thr Pro Pro Lys Ser Pro Ser Ser Ala Lys
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 8

(D) OTHER INFORMATION: /product= "OTHER"
/note= "phosphorylated Thr"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Lys Lys Val Ala Val Val Arg Thr Pro Pro Lys Ser Pro Ser Ser Ala Lys
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 12

(D) OTHER INFORMATION: /product= "OTHER"
/note= "phosphorylated Ser"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Lys Lys Val Ala Val Val Arg Thr Pro Pro Lys Ser Pro Ser Ser Ala Lys
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 8
- (D) OTHER INFORMATION: /product= "OTHER"
/note= "phosphorylated Thr"

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 12
- (D) OTHER INFORMATION: /product= "OTHER"
/note= "phosphorylated Ser"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Lys Lys Val Ala Val Val Arg Thr Pro Pro Lys Ser Pro Ser Ser Ala Lys
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: unknown

40

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Ala	Glu	Ile	Val	Tyr	Lys	Ser	Pro	Val	Val	Ser	Gly	Asp	Thr	Ser	Pro	Arg	His	Leu
1					5				10					15				

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 7

(D) OTHER INFORMATION: /product= "OTHER"
/note= "phosphorylated Ser"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Ala	Glu	Ile	Val	Tyr	Lys	Ser	Pro	Val	Val	Ser	Gly	Asp	Thr	Ser	Pro	Arg	His	Leu
1					5				10					15				

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 11

(D) OTHER INFORMATION: /product= "OTHER"
/note= "phosphorylated Ser"

41

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Ala Glu Ile Val Tyr Lys Ser Pro Val Val Ser Gly Asp Thr Ser Pro Arg His Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 14

(D) OTHER INFORMATION: /product= "OTHER"

/note= "phosphorylated Thr"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Ala Glu Ile Val Tyr Lys Ser Pro Val Val Ser Gly Asp Thr Ser Pro Arg His Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 15

(D) OTHER INFORMATION: /product= "OTHER"

/note= "phosphorylated Ser"

42

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Ala Glu Ile Val Tyr Lys Ser Pro Val Val Ser Gly Asp Thr Ser Pro Arg His Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 7
- (D) OTHER INFORMATION: /product= "OTHER"
/note= "phosphorylated Ser"

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 11
- (D) OTHER INFORMATION: /product= "OTHER"
/note= "phosphorylated Ser"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Ala Glu Ile Val Tyr Lys Ser Pro Val Val Ser Gly Asp Thr Ser Pro Arg His Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

43

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 7

(D) OTHER INFORMATION: /product= "OTHER"

/note= "phosphorylated Ser"

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 14

(D) OTHER INFORMATION: /product= "OTHER"

/note= "phosphorylated Thr"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Ala Glu Ile Val Tyr Lys Ser Pro Val Val Ser Gly Asp Thr Ser Pro Arg His Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 7

(D) OTHER INFORMATION: /product= "OTHER"

/note= "phosphorylated Ser"

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 15

(D) OTHER INFORMATION: /product= "OTHER"

/note= "phosphorylated Ser"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Ala Glu Ile Val Tyr Lys Ser Pro Val Val Ser Gly Asp Thr Ser Pro Arg His Leu
1 5 10 15

44

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 11
- (D) OTHER INFORMATION: /product= "OTHER"
/note= "phosphorylated Ser"

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 14
- (D) OTHER INFORMATION: /product= "OTHER"
/note= "phosphorylated Thr"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Ala Glu Ile Val Tyr Lys Ser Pro Val Val Ser Gly Asp Thr Ser Pro Arg His Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 11
- (D) OTHER INFORMATION: /product= "OTHER"
/note= "phosphorylated Ser"

45

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 15

(D) OTHER INFORMATION: /product= "OTHER"

/note= "phosphorylated Ser"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Ala	Glu	Ile	Val	Tyr	Lys	Ser	Pro	Val	Val	Ser	Gly	Asp	Thr	Ser	Pro	Arg	His	Leu
1					5				10					15				

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 14

(D) OTHER INFORMATION: /product= "OTHER"

/note= "phosphorylated Thr"

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 15

(D) OTHER INFORMATION: /product= "OTHER"

/note= "phosphorylated Ser"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Ala	Glu	Ile	Val	Tyr	Lys	Ser	Pro	Val	Val	Ser	Gly	Asp	Thr	Ser	Pro	Arg	His	Leu
1					5				10					15				

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 amino acids

(B) TYPE: amino acid

46

(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Modified-site
(B) LOCATION: 5
(D) OTHER INFORMATION: /product= "OTHER"
/note= "phosphorylated Ser"

(ix) FEATURE:

(A) NAME/KEY: Modified-site
(B) LOCATION: 7
(D) OTHER INFORMATION: /product= "OTHER"
/note= "phosphorylated Ser"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Ala Asp Glu Val Ser Ala Ser Leu Ala Lys Gln Gly Leu
1 5 10

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Modified-site
(B) LOCATION: 6
(D) OTHER INFORMATION: /product= "OTHER"
/note= "phosphorylated Ser"

(ix) FEATURE:

(A) NAME/KEY: Modified-site
(B) LOCATION: 11
(D) OTHER INFORMATION: /product= "OTHER"
/note= "phosphorylated Ser"

47

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Glu	Glu	Lys	Gly	Lys	Ser	Pro	Val	Pro	Lys	Ser	Pro	Val	Glu	Glu	Lys	Gly
1				5					10					15		

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Ala	Val	Tyr	Thr	Arg	Ile	Met	Met	Asn	Gly	Gly	Arg	Leu	Lys	Arg
1					5				10					15

What is claimed is:

1. A synthetic multiphosphorylated peptide derived from the τ protein of the paired helical filaments which is useful as an antigen for preparing an antibody specific for paired helical filaments associated with Alzheimer's disease.
2. The peptide according to claim 1, wherein the peptide contains two phosphorylated amino acids.
3. The peptide according to claim 1, wherein the phosphorylated amino acids are serines and threonines, which are separated by between 1 - 6 amino acids.
4. The peptide according to claim 1, wherein the peptide is derived from a fragment of the τ protein consisting of amino acids about 207 to about 222, amino acids about 224 to about 240, and amino acids about 390 to about 408.
5. The peptide according to claim 4, wherein the fragment consists of amino acids 207-222 of τ and the peptide is selected from the group consisting of:
 - (a) GSRSR(T)P(S)LPTPPTRE, SEQ ID NO:5;
 - (b) GSRSR(T)PSLP(T)PPTRE, SEQ ID NO:6; and
 - (c) GSRSRTP(S)LP(T)PPTRE, SEQ ID NO: 7, wherein the () indicates a phosphorylated amino acid.
6. The peptide according to claim 4, wherein the fragment consists of amino acids 224-240 of τ and the peptide is KKVAVVR(T)PPK(S)PSSAK, SEQ ID NO:11, wherein () indicates a phosphorylated amino acid.

7. The peptide according to claim 4, wherein the fragment consists of amino acids 390-408 of τ and the peptide is selected from the group consisting of:

- (a) AEIVYK(S)PVV(S)GDTSPRHL, SEQ ID NO:17;
- (b) AEIVYK(S)PVVSGD(T)SPRHL, SEQ ID NO:18;
- (c) AEIVYK(S)PVVSGDT(S)PRHL, SEQ ID NO:19;
- (d) AEIVYKSPVV(S)GD(T)SPRHL, SEQ ID NO:20;
- (e) AEIVYKSPVV(S)GDT(S)PRHL, SEQ ID NO:21;
- (f) AEIVYKSPVVS GD(T)(S)PRHL, SEQ ID NO:22, wherein ()

indicates a phosphorylated amino acid.

8. A method for generating an antibody useful in the specific diagnosis of Alzheimer's disease, comprising the step of:

administering to a mammal a synthetic multiphosphorylated peptide derived from the τ protein of the paired helical filaments which is useful as an immunogen for preparing an antibody specific for paired helical filaments associated with Alzheimer's disease.

9. The method according to claim 8 wherein the mammal is a mouse.

10. The method according to claim 8 wherein the antibody is a monoclonal antibody or a fragment thereof.

11. The method according to claim 8 wherein the mammal is a rabbit.

12. The method according to claim 8 wherein the antibody is a polyclonal antibody.

13. The method according to claim 8, wherein the peptide contains two phosphorylated amino acids.

14. The method according to claim 8, wherein the phosphorylated amino acids are serines and threonines which are separated by between 1 and 6 amino acids.

15. The method according to claim 8, wherein the peptide is derived from a fragment of the τ protein consisting of amino acids about 207 to about 222, amino acids about 224 to about 240 and amino acids about 390 to about 408.

16. The method according to claim 15, wherein the fragment is amino acids 207-222 of τ and the peptide is selected from the group consisting of:

- (a) GSRSR(T)P(S)LPTPPTRE, SEQ ID NO:5;
- (b) GSRSR(T)PSLP(T)PPTRE, SEQ ID NO:6; and
- (c) GSRSRTP(S)LP(T)PPTRE, SEQ ID NO:7; wherein the () indicates a phosphorylated amino acid.

17. The method according to claim 15, wherein the fragment is amino acids 224-240 of τ and the peptide is peptide is KKVAVVR(T)PPK(S)PSSAK, SEQ ID NO:11, wherein () indicates a phosphorylated amino acid.

18. The method according to claim 15, wherein the fragment is amino acids 390-408 of τ and the peptide is selected from the group consisting of:

- (a) AEIVYK(S)PVV(S)GDTSPRHL, SEQ ID NO:17;
- (b) AEIVYK(S)PVVSGD(T)SPRHL, SEQ ID NO:18;
- (c) AEIVYK(S)PVVSGDT(S)PRHL, SEQ ID NO:19;
- (d) AEIVYKSPVV(S)GD(T)SPRHL, SEQ ID NO:20;
- (e) AEIVYKSPVV(S)GDT(S)PRHL, SEQ ID NO:21; and

(f) AEIVYKSPVVS_{GD}(T)(S)PRHL, SEQ ID NO:22; wherein () indicates a phosphorylated amino acid.

19. An antibody generated according to the method of claim 8, which is characterized by for the τ protein of the paired helical filaments associated with Alzheimer's disease.

20. An antibody or fragment thereof, which is specific for a synthetic multiphosphorylated peptide according to claim 1, said antibody or antibody fragment selected from the group consisting of:

- (a) a monoclonal antibody;
- (b) a polyclonal antibody;
- (c) a recombinant antibody; and
- (d) Fab, Fv, or F(ab')₂ of any of (a) to (c).

21. The antibody according to claim 20, wherein said recombinant antibody is selected from the group consisting of bifunctional antibodies, chimeric antibodies and humanized antibodies.

22. A method for identifying compounds useful in the treatment and diagnosis of Alzheimer's disease comprising the steps of:

(a) contacting a synthetic multiphosphorylated peptide according to claim 1 with a test compound to permit binding of the test compound to the peptide; and

(b) determining the amount of test compound which is bound to the peptide, wherein specific binding of the compound to the peptide indicates that the test compound is capable of being used for treatment or diagnosis of Alzheimer's disease.

23. A method for identifying compounds useful in the treatment and diagnosis of Alzheimer's disease comprising the steps of:

(a) contacting a synthetic multiphosphorylated peptide according to claim 1 with a known target of said peptide and a test compound to permit binding; and

(b) determining the amount of target which is bound to the peptide, wherein inhibition of binding of target to the peptide indicates binding of the test compound to the peptide, indicating that the test compound is capable of being used for treatment or diagnosis of Alzheimer's disease.

24. The method according to claim 22 or 23, wherein the synthetic multiphosphorylated peptide is immobilized on a solid support.

25. A compound identified by the method of any of claims 22 to 24.

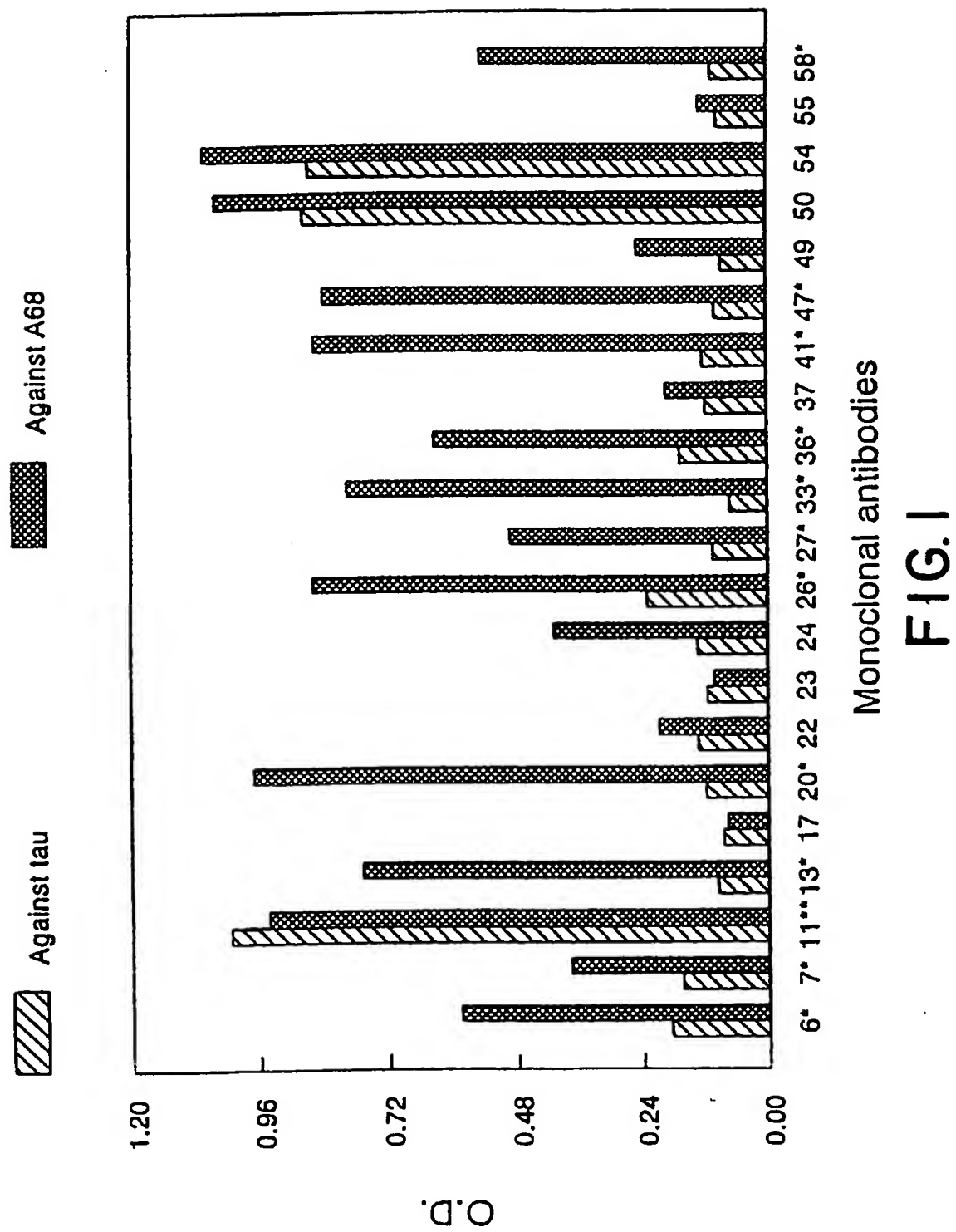
26. A method of treating a patient with Alzheimer's disease comprising the step of administering to the patient an antibody or fragment thereof according to any of claims 19 to 21.

27. A method of treating a patient with Alzheimer's disease comprising the step of administering to the patient a multiphosphorylated peptide according to claim 1.

28. Use of an antibody according to any of claims 8 or 19 to 21 in the preparation of a medicament for treatment of Alzheimer's disease.

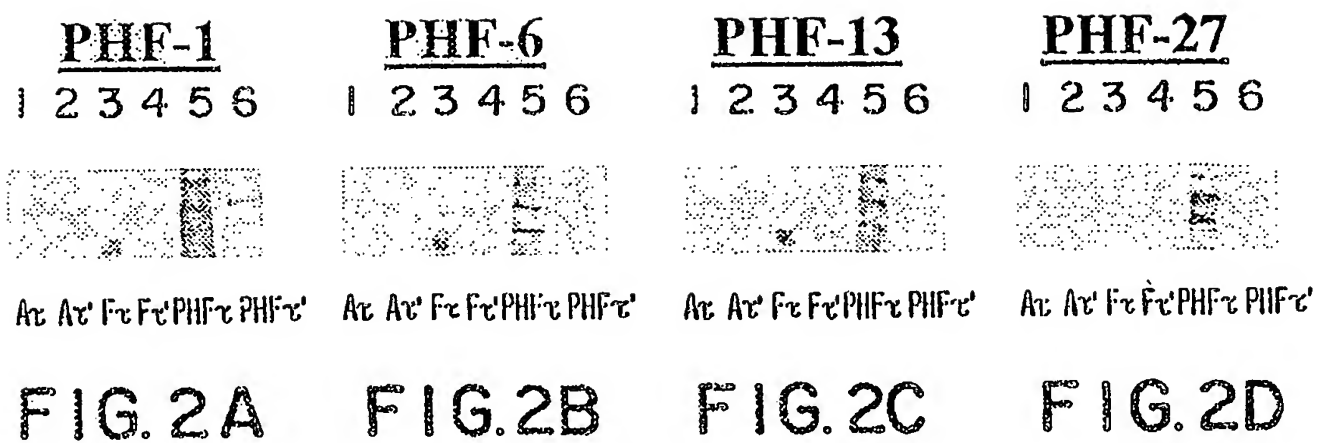
29. Use of a multiphosphorylated peptide according to claim 1 in the preparation of a medicament for treatment of Alzheimer's disease.

1/15



Monoclonal antibodies

FIG. I



3/15

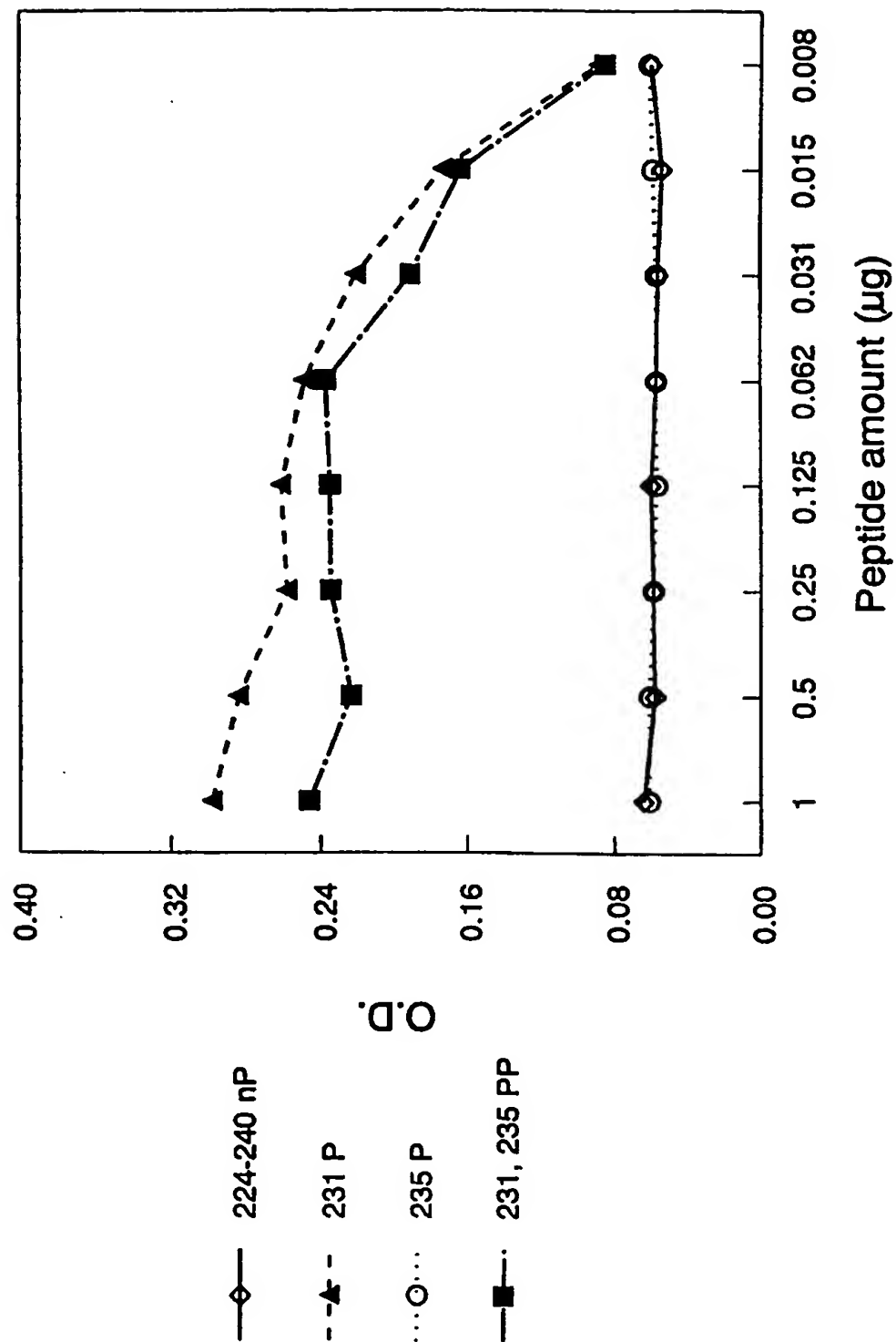


FIG. 3

4/15

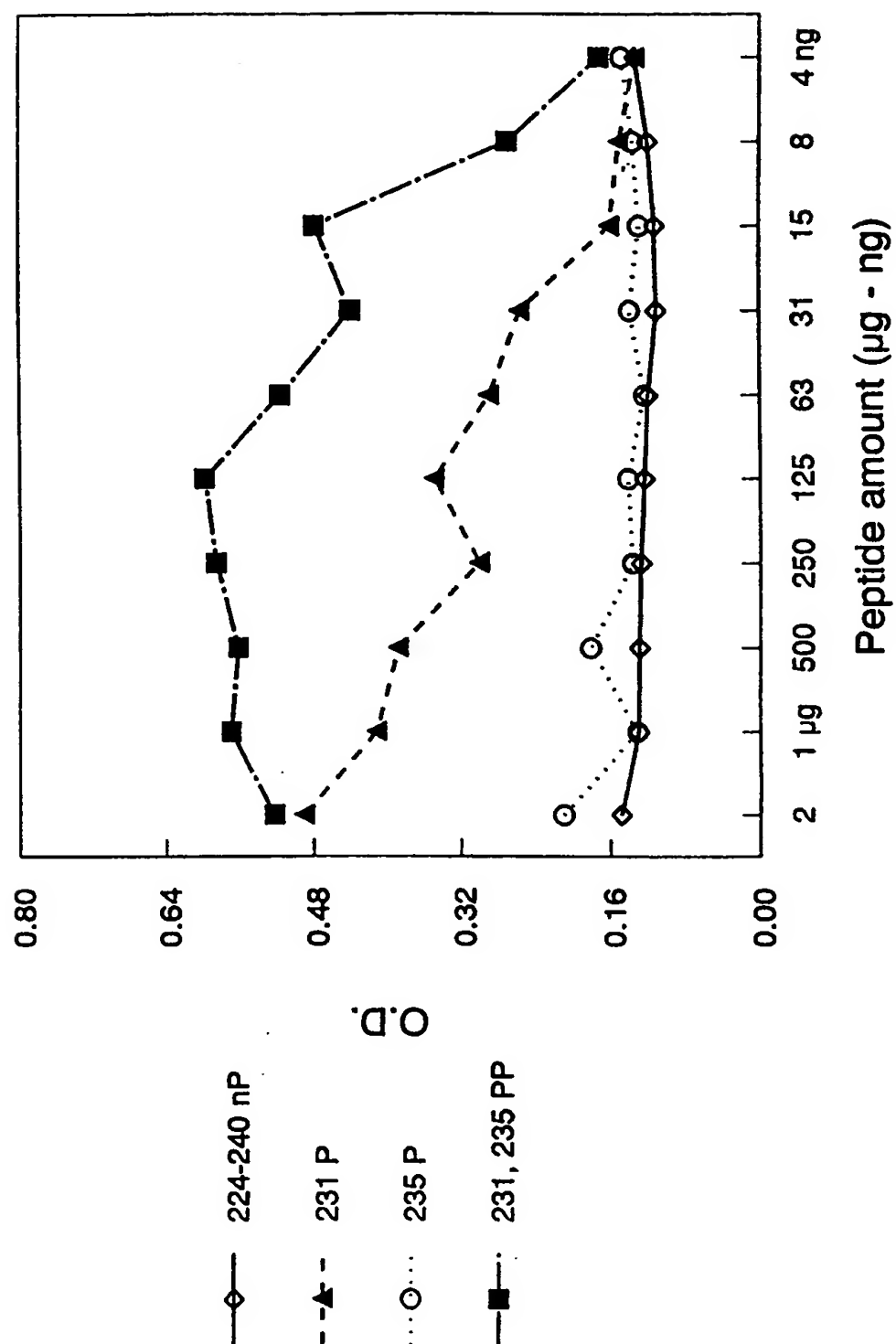


FIG. 4

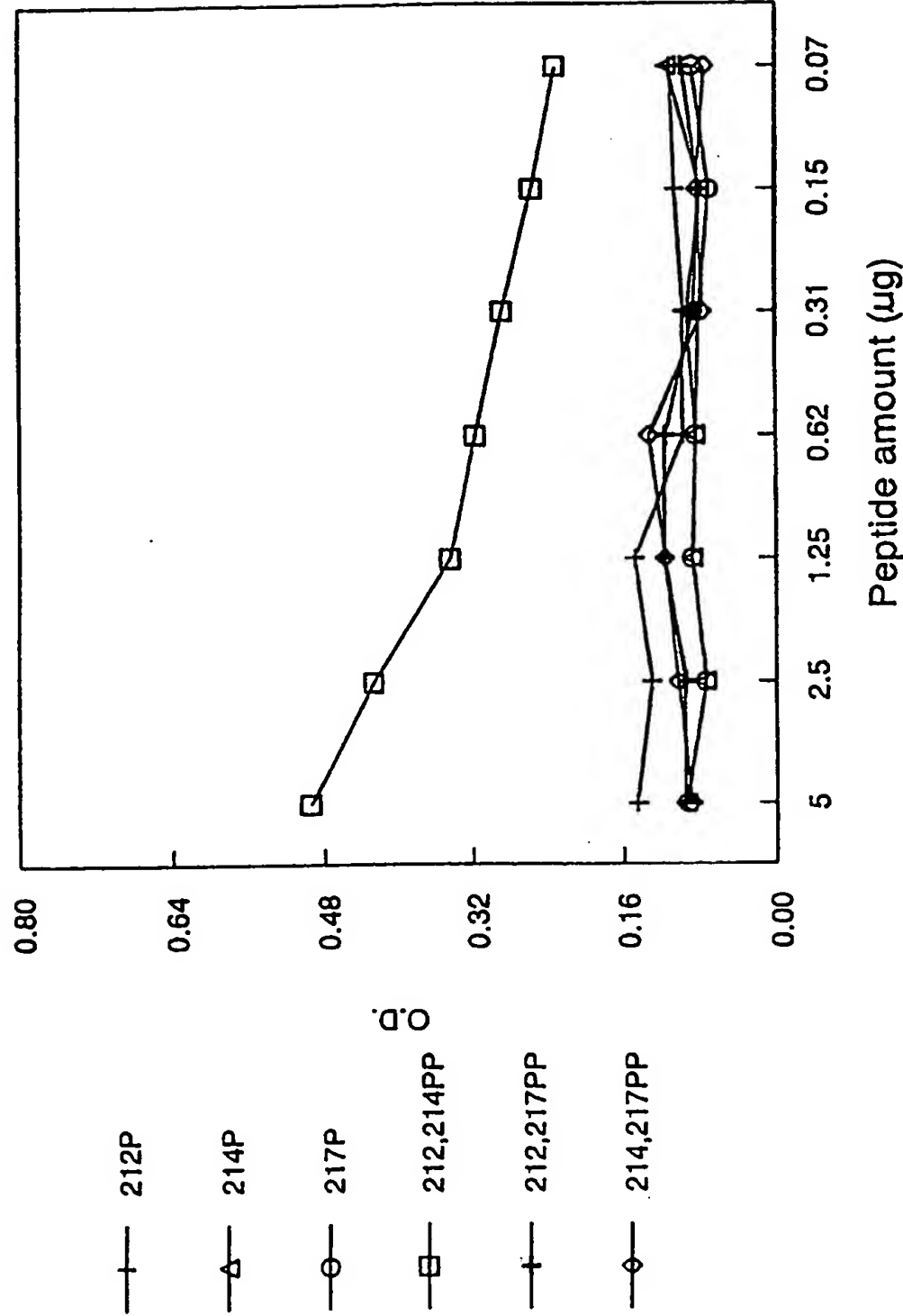


FIG. 5

6/15

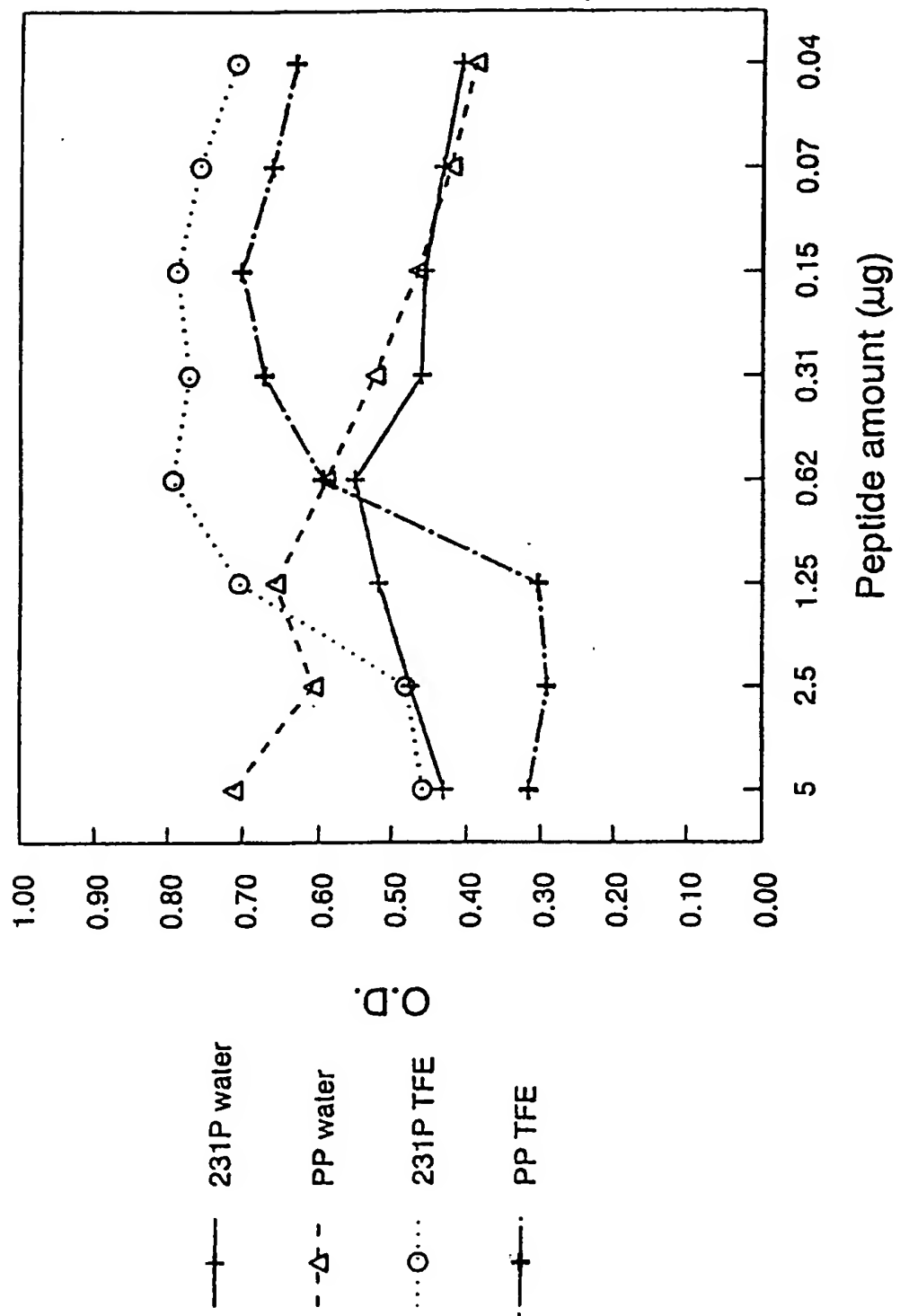


FIG. 6A

7/15

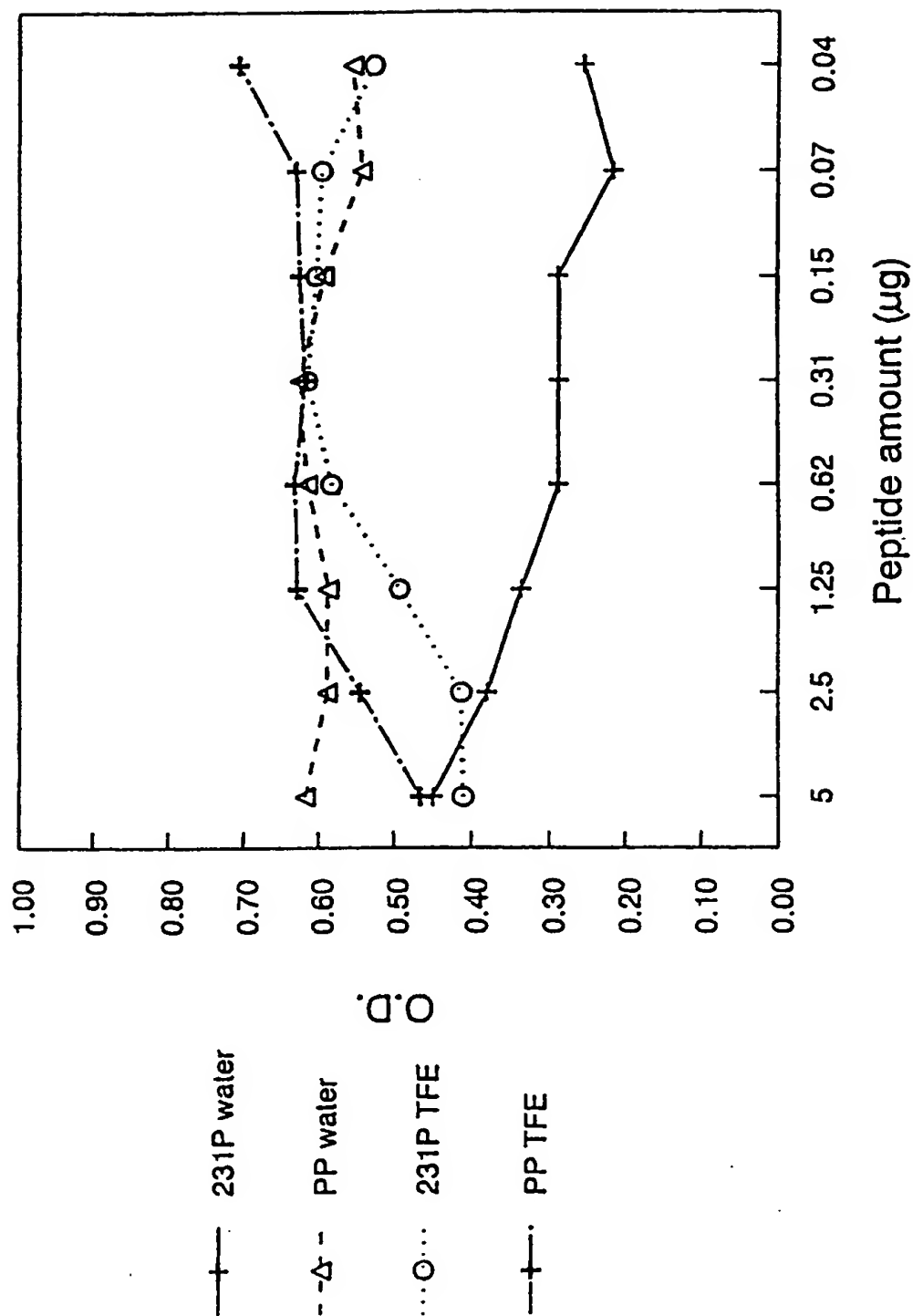


FIG. 6B

8/15

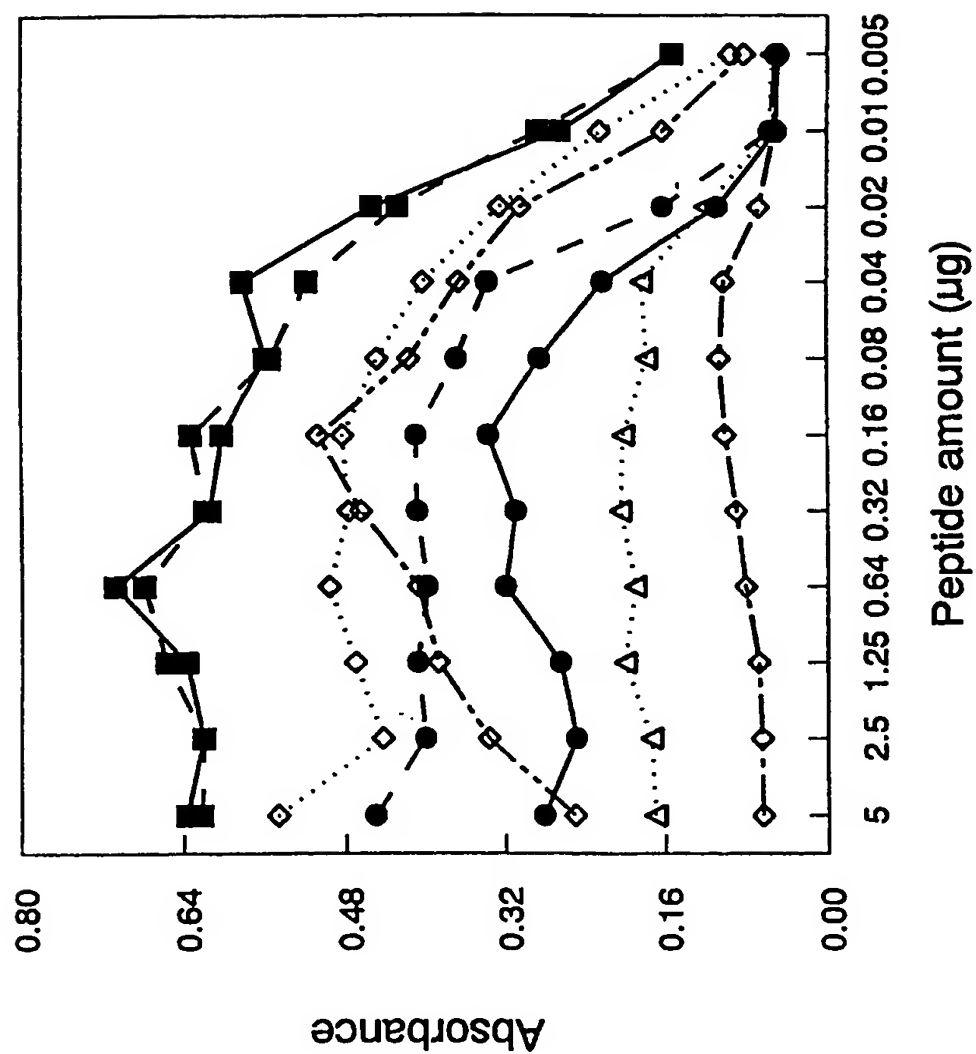


FIG. 7

9/15

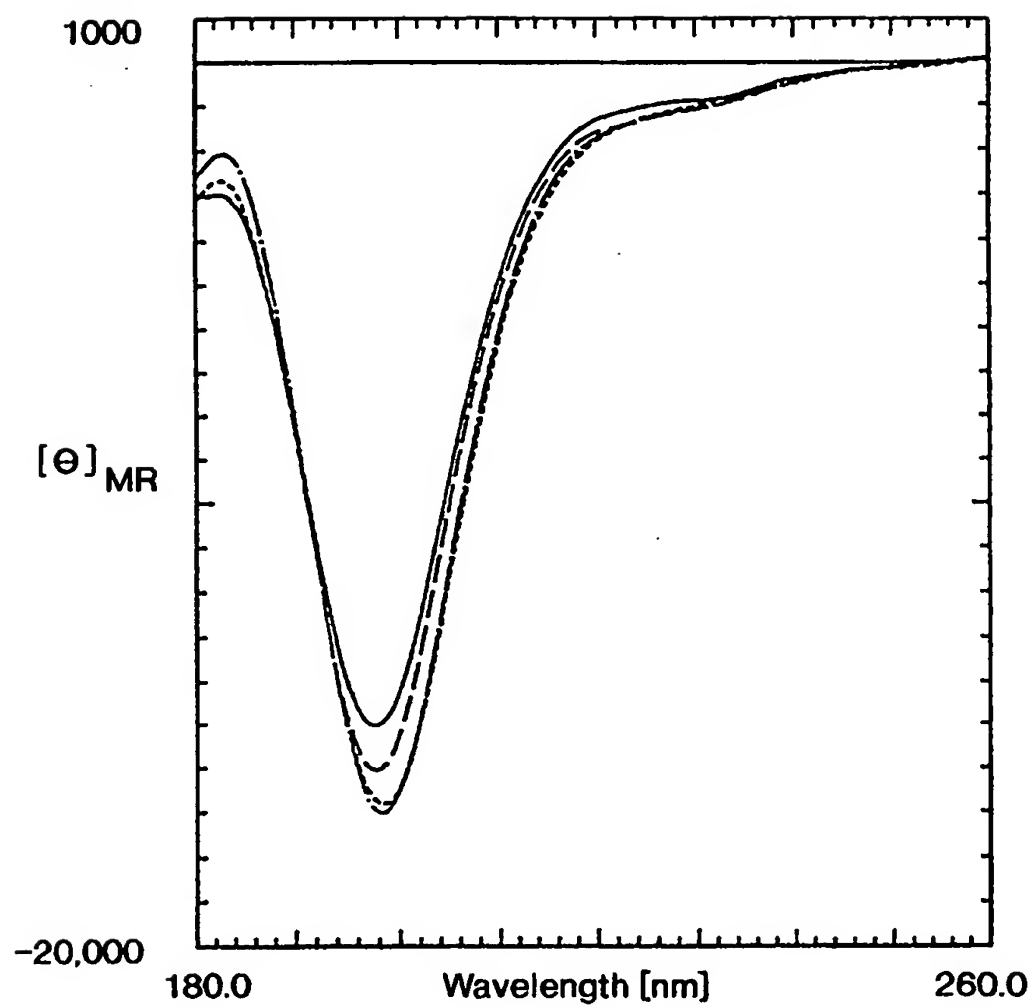


FIG. 8A

10/15

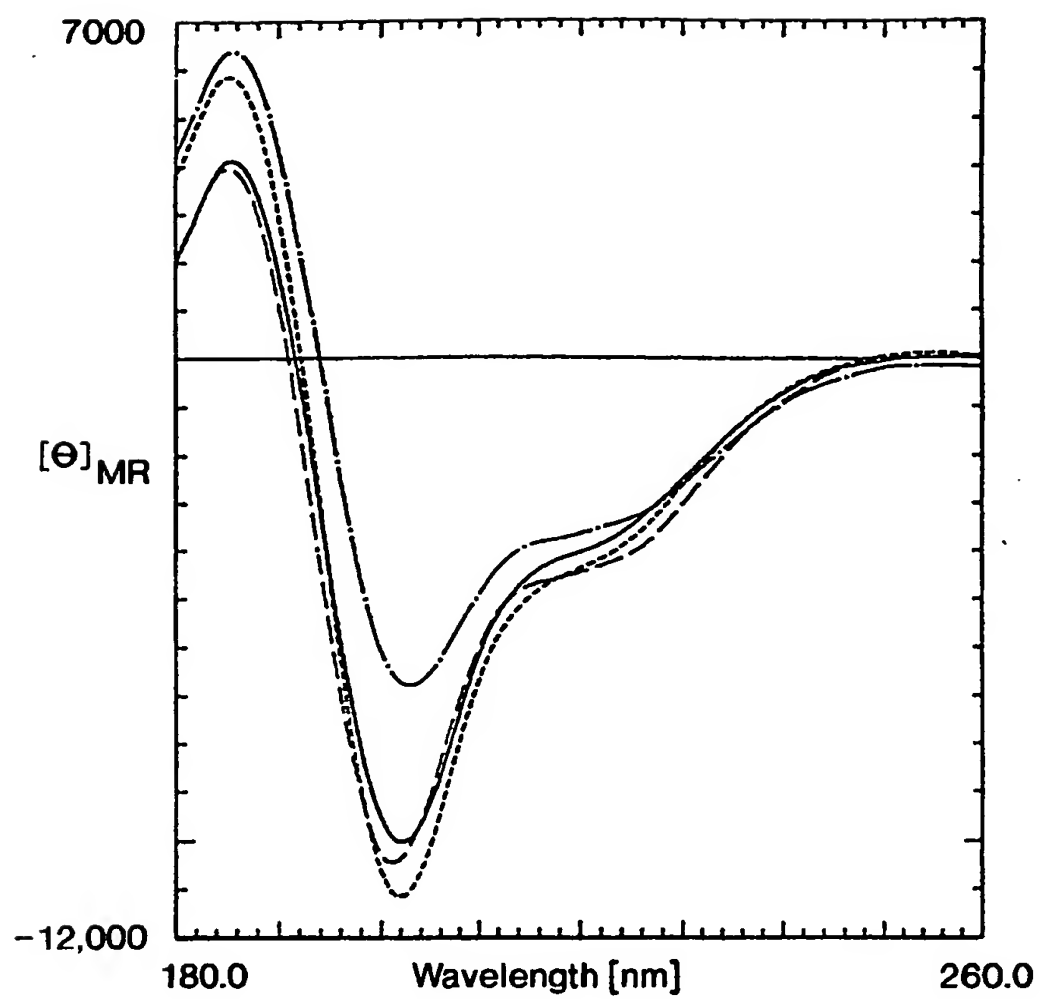


FIG. 8B

11/ 15

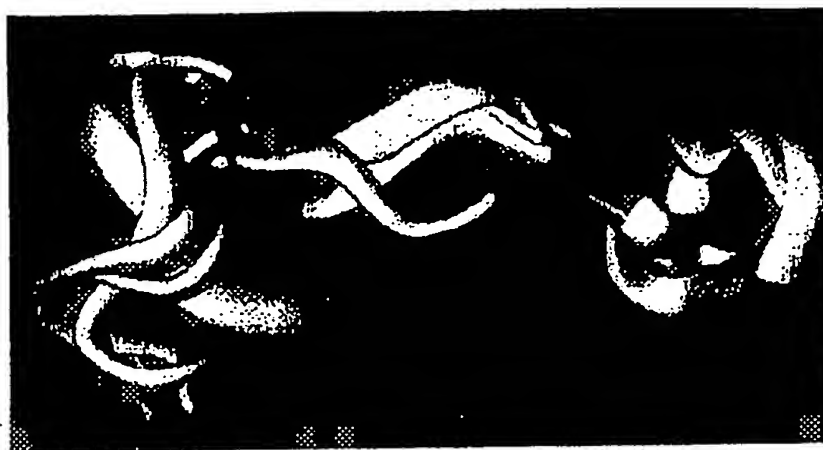


FIG. 9A



FIG. 9B

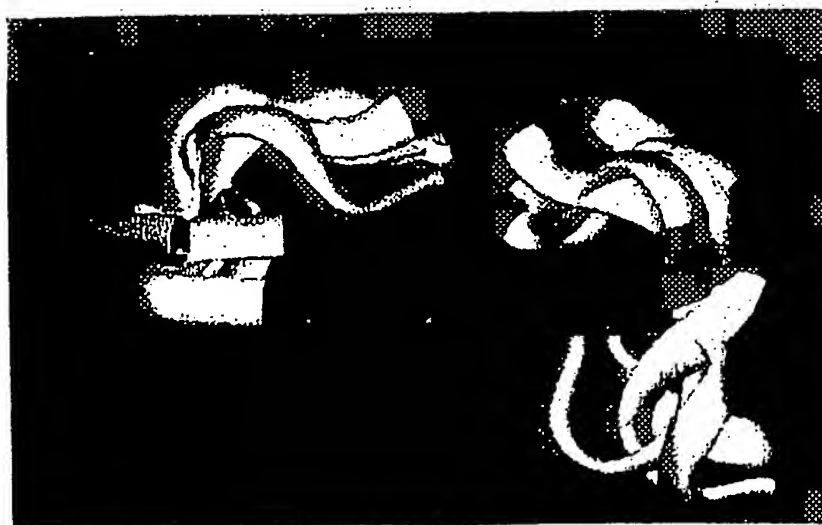


FIG. 9C

12/15

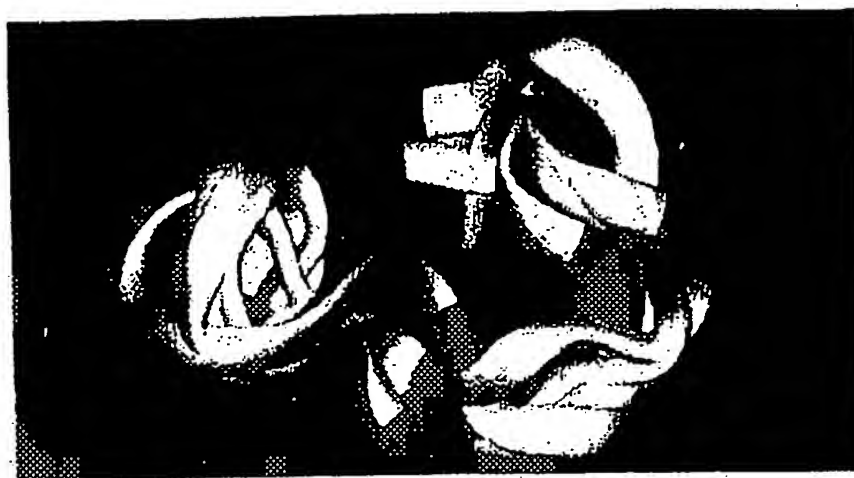


FIG. 9D

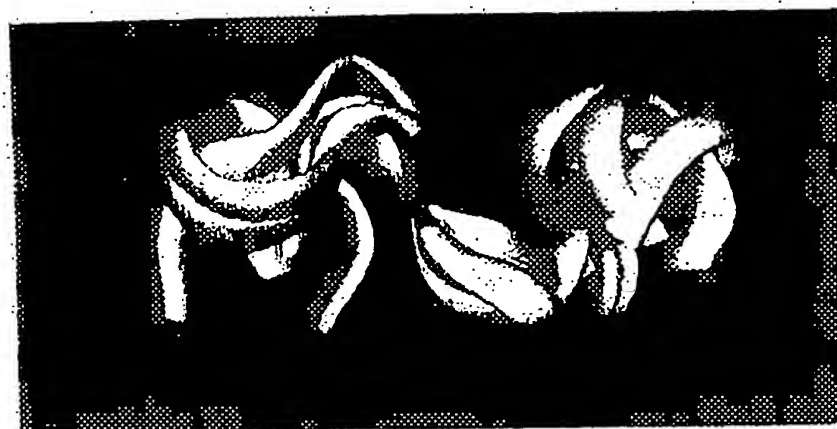
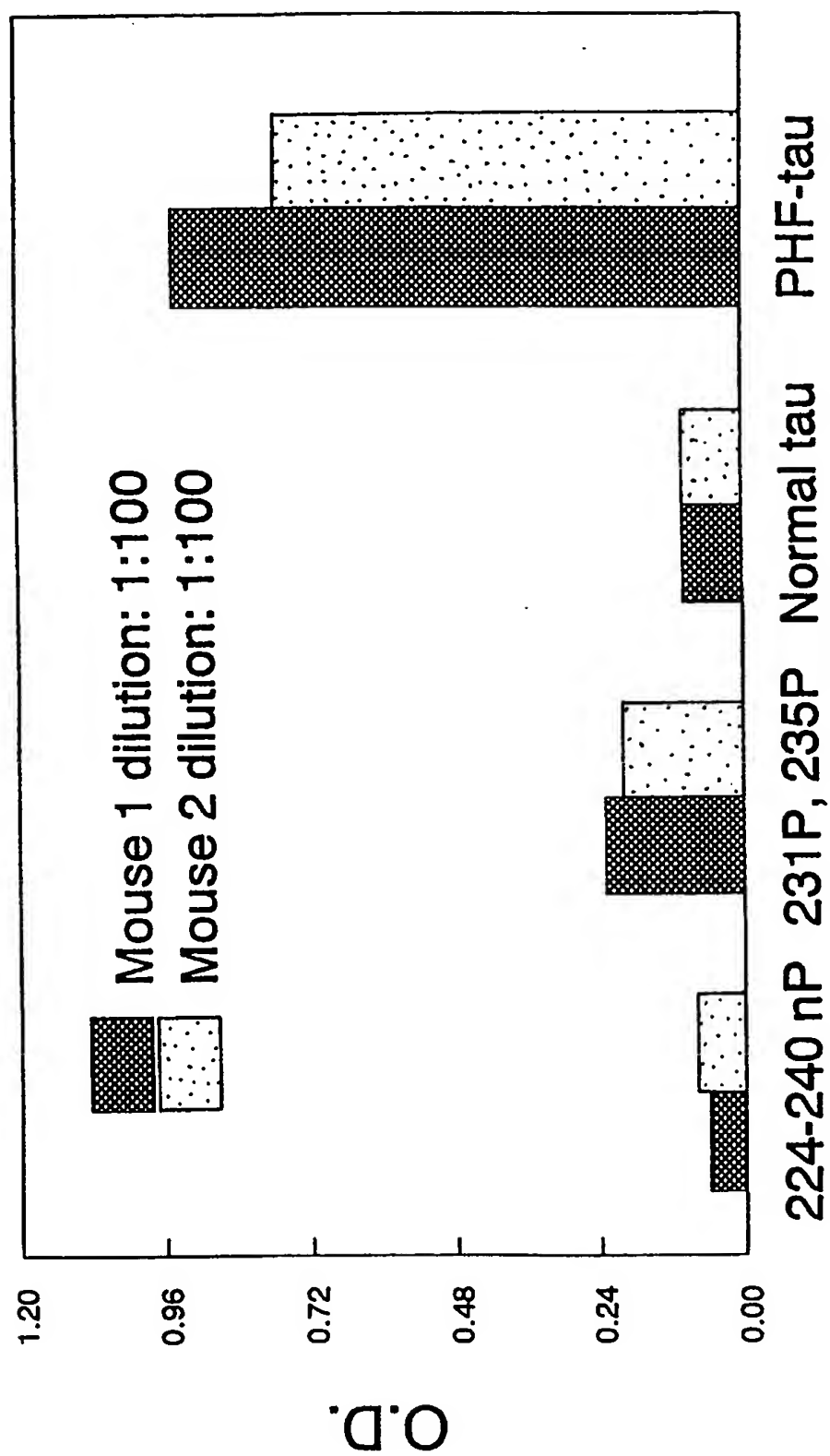


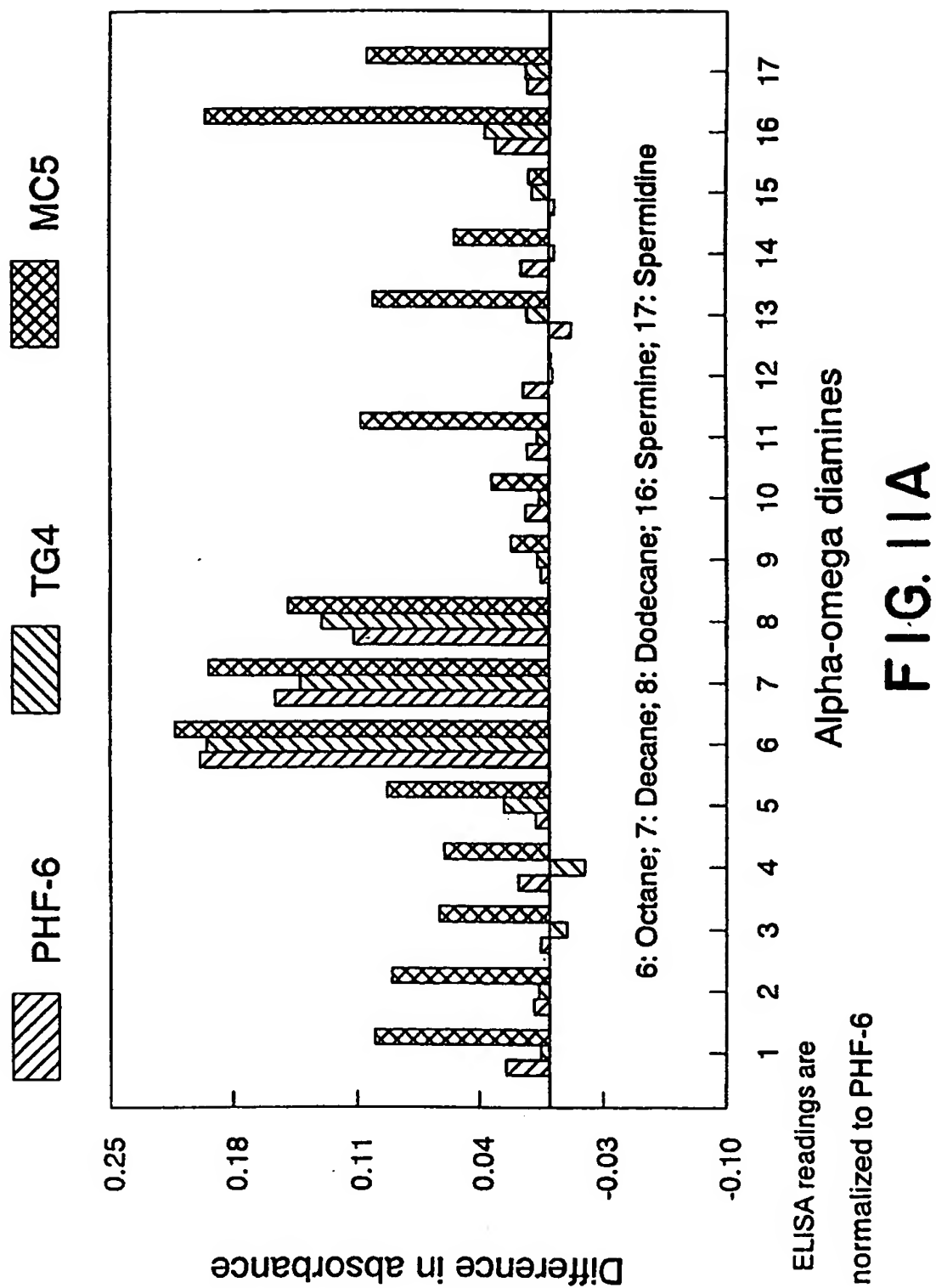
FIG. 9E

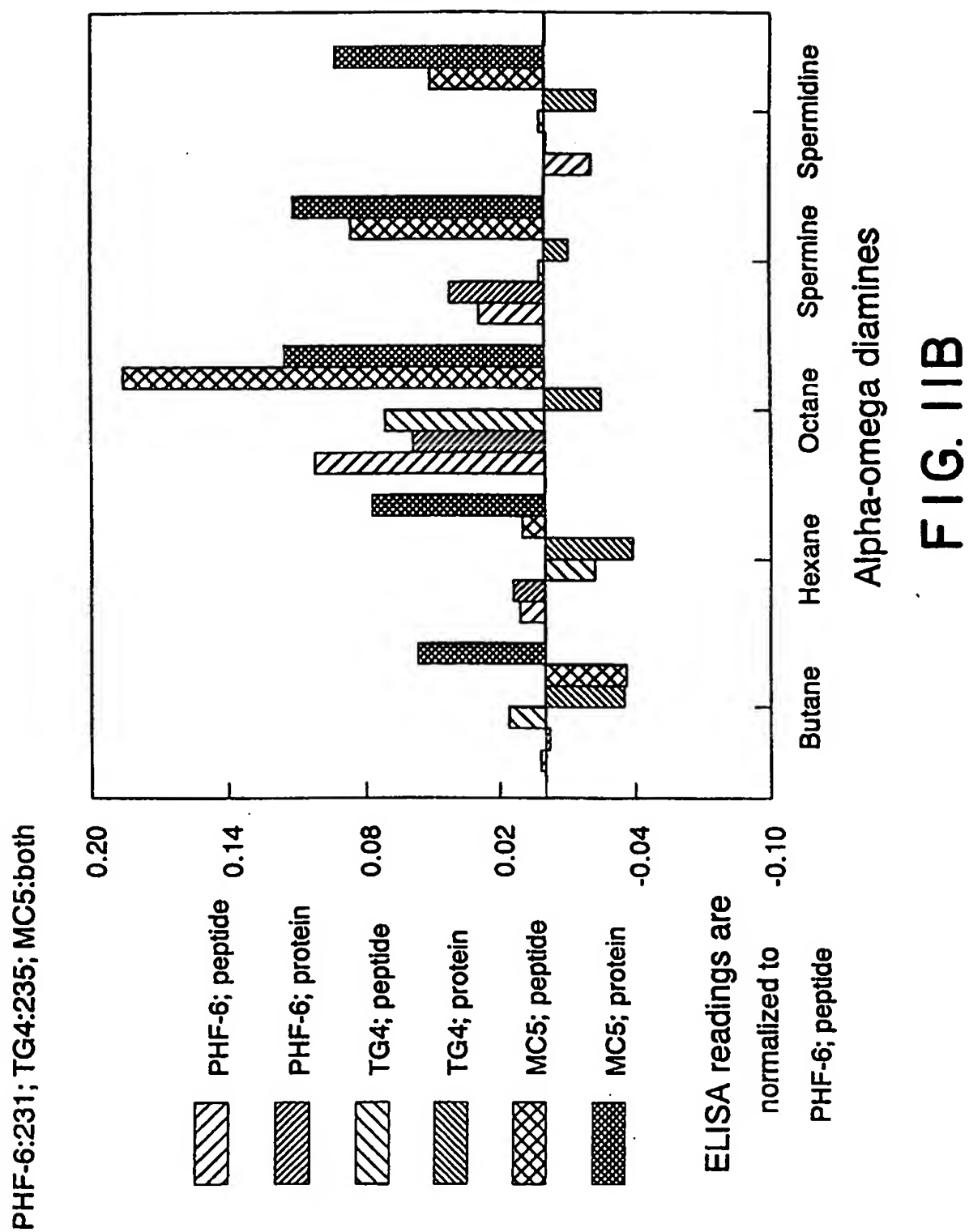


FIG. 9F



Antigens
FIG. 10





INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/21116

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : 530/300, 387.1; 435/4

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/300, 387.1; 435/4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG; author and word. Search terms include: tau, phosphory?, antibod?, serine io threonine

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ZEMLAN et al. Monoclonal antibody PHF-9 recognizes phosphorylated serine 404 of tau protein and labels paired helical filaments. Journal of Neuroscience Research. October 1996. Vol. 46, No. 1. pages 90-97, Abstract only.	1-29
Y	SHIURBA et al. Immunohistochemcistry of tau phophoserine 413 and tau protein kinase I in Alzheimer pathology. Brain Research. October 1996. Vol. 737, No. 1-2. pages 119-132, Abstract only.	1-29
Y	Kimura et al. Sequential cahnges of tau-site-specific phosphorylation during development of paired helical filaments. Dememtia. July-August 1996. Vol. 7, No. 4. pages 177-181, Abstract only.	1-29



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

25 FEBRUARY 1998

Date of mailing of the international search report

20 MAR 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

HEATHER BAKALYAR

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/21116

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

A61K 38/00; C07K 2/00, 4/00, 5/00, 7/00, 14/00, 16/00, 17/00; C12Q 1/00



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 38/00, C07K 2/00, 4/00, 5/00, 7/00, 14/00, 16/00, 17/00, C12Q 1/00	A1	(11) International Publication Number: WO 98/22120 (43) International Publication Date: 28 May 1998 (28.05.98)
(21) International Application Number: PCT/US97/21116 (22) International Filing Date: 19 November 1997 (19.11.97) (30) Priority Data: 60/031,169 19 November 1996 (19.11.96) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 60/031,169 (CIP) Filed on 19 November 1996 (19.11.96) (71) Applicants (for all designated States except US): THE WISTAR INSTITUTE OF ANATOMY & BIOLOGY [US/US]; 3601 Spruce Street, Philadelphia, PA 19104 (US). TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA [US/US]; Suite 300, 3700 Market Street, Philadelphia, PA 19104 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): OTVOS, Laszlo [US/US]; 801 Mockingbird Lane, Audubon, PA 19403 (US). HOFFMANN, Ralf [DE/US]; 6812 Drexelbrook Drive, Drexel Hill, PA 19026 (US). LEE, Virginia, M-Y. [US/US]; 2005 Pine Street, Philadelphia, PA 19103 (US).	(74) Agents: KODROFF, Cathy, A. et al.; Howson and Howson, Spring House Corporate Center, P.O. Box 457, Spring House, PA 19477 (US). (81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(54) Title: DIAGNOSTIC AND THERAPEUTIC REAGENTS FOR ALZHEIMER'S DISEASE (57) Abstract <p>A synthetic multiphosphorylated peptide derived from the τ protein of the paired helical filaments is described. The Alzheimer disease specificity is provided by the presence of phosphate moieties on nearby serine and threonine residues, and the reverse-turn structure of the multiphosphorylated protein fragment. The multiphosphorylated peptide is useful as an antigen and a binding partner for identifying inhibitor compounds which interact with the peptide and the hyperphosphorylated tau protein, including Alzheimer's specific antibodies. The resulting antibodies are useful diagnostically and therapeutically. The inhibitors that specifically bind to multiphosphorylated tau peptides and proteins are useful for eliminating abnormally hyperphosphorylated tau.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		